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## Spanish success story

Workers' co-ops have taken root in the Basque country; could they in Britain?

FEW would disagree these days that large parts of British industry are in serious decline, and that in some cases this decline may be irreversible. Almost everything has, at one time or another, been blamed for this, but there is little doubt that one crippling aspect in many industries is the immense distrust that exists between management and the workforce. Indeed, many of those involved in the pitched battles which occupy the headlines day after day speak as if conflict is a necessary ingredient in industrial relations. Those driven to despair by arguments over the sizes of the slices being cut from a rapidly shrinking cake may be interested to know that a totally different form of industrial structure has been quietly constructed in the Basque provinces of Spain in the last twenty years; and it shows signs of considerable health. It is the workers' co-operative movement centred on Mondragon, about 450 kilometres from Bilbao.

Basque Spain is a surprisingly industrialised area. There are numerous towns and villages with all manner of industry from the lightest to the heaviest, much of it blithely producing extraordinarily high levels of environmental pollution. A Basque priest, José Maria Arizmendi, came to Mondragon, an economically devastated and repressed town in the midst of this industrial area, in 1941. His first job was to try to improve secondary and technical education which was at that time minimal. As educational standards gradually rose, he and some of his first students started to ask whether they could not participate more directly in industrial enterprises than merely as employees of capitalist firms. Out of these discussions came the first workers' co-operative, ULGOR, in 1956. A manufacturer of domestic appliances, it began with 23 people; it now employs around 3,500. In the past twenty years, more than sixty co-operatives have been founded, and they employ around 15,000 people. They range from agricultural co-ops through to iron and steel foundries. The unifying feature is that no employee can join without taking a stake (of between £1,000 and £2,000) in the enterprise. Basques are traditionally savers and there is said to be no shortage of applicants.

Although management of the co-ops is ultimately in the

hands of the whole work force, care is taken to give managers many of the freedoms of their capitalist counterparts to run their co-ops imaginatively. They do not, however, have the freedom to sack employees. Salary levels for each job are publicly established, and there is only a ratio of 3:1 between the top and bottom salary rates. Those retiring from co-ops now are receiving at least £10,000 in addition to their pension.

It is not just the workers who co-operate — co-operatives themselves all work closely together. The most striking example is the communally owned bank, Caja Laboral Popular, which acts both as a conventional bank for the workers and as a very sophisticated merchant bank for the support of existing co-ops and the establishment of new ones. Other examples of so-called second-degree co-operatives include schools, a social security organisation and a research and development centre. There is no doubt that most of the co-operatives have been successful in conventional economic terms, in addition to providing an apparently happy working environment in which there is an openness in all activities. Managers speak enthusiastically of the common purpose in their factories, the willingness to work all hours to get satisfied customers and the high level of productivity.

Could Mondragon-type co-operatives survive in Britain? In the past few years the handful of older co-ops in Britain, many with structures very unlike those at Mondragon, have been joined, with maximum publicity, by co-ops encouraged by Mr Tony Benn. The trouble is that they have only become co-operatives as a last, desperate resort when a capitalist venture has failed. And indeed one, the *Scottish Daily News*, soon folded. The Mondragon experience points quite unambiguously to the importance of local workers coming together, probably on a small scale to start with, with their own ideas for something entirely new. □

● Two valuable books on worker co-ops are: *Worker-Owners* (Anglo-German Foundation, St Stephen's House, Victoria Embankment, London SW1; £2.90) and *The Case for Workers' Co-ops*, by Robert Oakeshott (Routledge & Kegan Paul; £7.95).



# Shuttle problems delay next planetary mission

**David Dickson** reports from Washington on why Jupiter must wait

The US National Aeronautics and Space Administration announced last week that it intends to delay the launch of Project Galileo — a two-vehicle mission to the planet Jupiter — from 1982 to 1984 because of problems in the development of the space shuttle.

The two-year delay will also mean separate launches for the two vehicles, one of which will orbit the planet, and the other descend into its atmosphere; initially plans were to use a single launch, but the delay means that the spacecraft will no longer be able to make use of extra push given by the planet Mars.

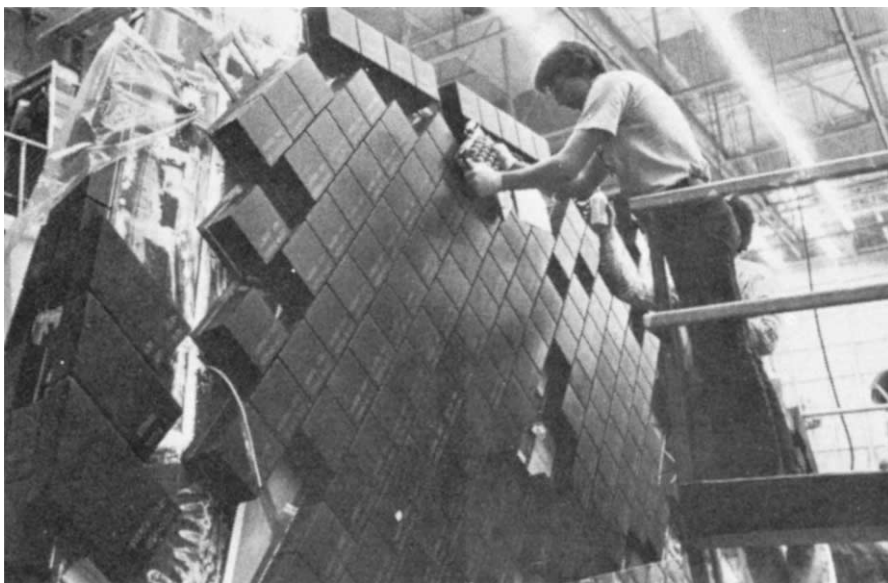
Dr Thomas Mutch, associate administrator for space science, told a House subcommittee in Washington last week that the agency had studied the implications of delays in developing the shuttle engines and decided that the risks involved in meeting the necessary schedules for both the shuttle and the inertial upper stage from which Galileo will be launched were "unacceptable". "The most cost-effective and least-risk approach to retain the Galileo scientific objectives is to delay the launch until 1984, and launch the orbiter and the probe separately", Dr Mutch said.

The delay, and the need for a double launch, will mean a considerable increase in the cost of the project. Originally, with a 1982 launch, this was estimated at about \$450 million; the new estimate, including in particular the extra hardware which the double launch will require, is \$675 million.

Dr Mutch told *Nature* that the new plans, which will involve launching the orbiter several weeks ahead of the probe, will not effect the scientific programmes, and could indeed increase the quality of the results. "Since there will be a separate carrier devoted to the probe, we can now optimise its trajectory to get the maximum coordination with the orbiter," Dr Mutch said.

However the delay could mean further problems for the Galileo programme from Congress, which will have to give its approval to the new schedule and the increased cost. Two years ago, a bid to kill the project by Representative Edward Boland, chairman of NASA's appropriations subcommittee, was defeated on the floor of the House, only after a massive letter-writing campaign by the academic community.

This year, given the delays with the launch, the House accepted an amendment to NASA's appropriations bill proposed by Mr Boland which would give the subcommittee power to veto any move to split the mission. This has been rejected by the Senate, however, and negotiations are now taking place to find an adequate wording to



*Sticky problem for the space shuttle: some of these protective tiles came off in flight*

meet the House's concern for a review of the new plans, but without giving either legislative body veto powers.

NASA scientists are worried that Congress may try to reduce the costs by splitting the launch, and sending one half of the mission — possibly the orbiter — at a later date. They emphasise that NASA has no plans to split the mission in this way.

Meanwhile Dr Robert Frosch, administrator of NASA, told the House subcommittee that NASA calculated a 50-50 chance that the space shuttle would have its first flight by next summer. The flight was to have taken place this spring, but has been postponed after a series of technical problems. The most recent was replacing some of the thermal tiles, used to protect the re-usable shuttle when it re-enters the earth's orbit, which came

unstuck during testing.

Admitting that this was a problem which "should have been clear sooner, but was not," Dr Frosch told the subcommittee that about 7% of the tiles so far tested had had to be replaced, implying a total of 2,400 out of the shuttle's 34,000 tiles.

Dr Frosch said that technical delays, in particular with the testing of engine performance, meant that the total cost of the shuttle development programme was now likely to be about 20% more than the initial estimate of \$5.15 billion at 1971 prices. He told the subcommittee that NASA had made a number of management changes after investigations to discover why the shuttle programme had run into problems at this stage. But he emphasised that a major factor had been serious underfunding of the programme early on. □

## IQ tests 'unconstitutional'

In what promises to be a landmark decision after a seven-year court battle, San Francisco's chief district court judge, Robert Peckham, has ruled that the use of standardised IQ tests to categorise children as mentally retarded discriminates against minorities and is thus unconstitutional.

The immediate effect will be to make permanent a state-wide injunction against these tests and to force state officials to re-evaluate black children previously identified as "educable mentally retarded".

The judge struck out at what he called the "grossly disproportionate enrolments of black children in the so-called 'educable mentally retarded' classes". Although black children make up only 9% of the state school population, some 23% of the children enrolled in these classes are black. Not only is this unconstitutional, Judge Peckham ruled, but it violates federal civil

rights laws and statutes dealing with education for the handicapped as well. He ordered state officials to "monitor and eliminate" the disproportionate placements. The complex case was launched in 1971 by civil rights groups acting on behalf of six black children in San Francisco — known only as Larry *et al.* The children had been placed in special classes because they had scored lower than 75 on IQ tests. When they were tested later by a group of black psychologists, their scores ranged from 17 to 38 points higher. State officials argued that the testing protocols and procedures used by the black psychologists violated accepted practice.

However, the case apparently was decided more on the basis of the general limitations of IQ tests rather than on the specific problems of these particular children. In testimony that ran for more than 10,000 pages, Peckham heard a wide



range of arguments over the validity and proper use of IQ tests. Interestingly, neither side took a firm stand on the issue of whether or not "intelligence" as so measured represents an in-born ability. Rather, attorneys for the children argued that the tests are culturally biased, while the state argued that the tests remain good predictors of school performance for blacks as well as white.

The judge said that, in his opinion, "many black children have been isolated, stigmatised and provided with inadequate education on the basis of unwarranted and impermissible assumptions". The tests, he said, had not been modified or validated for blacks, while "almost no experts now contend that IQ measures innate intelligence".

Beyond the issue of the tests, themselves, Peckham found that the special classes are designed to provide only a "limited, dead-end education for children who, while not severely retarded, are incapable of mastering the skills taught in a regular curriculum". Since placement is assumed to be permanent, he said, "the children assigned to those classes are unlikely ever to succeed in school, even if they are not truly retarded."



Black pupils: tests discriminate against them

Just what the ultimate effect of this ruling may be remains unclear. Despite the injunction, enrolment of blacks in mentally retarded classes remains disproportionately high. IQ tests can still be used to identify gifted children and those that qualify for some other special programmes. An appeal may be launched and related cases are pending in other parts of the country.

The case brought into full public view many of the problems related to IQ testing that have been argued about in scientific circles for many years. And there may be a growing sentiment in favour of paying more attention to "performance" rather than "ability". In an editorial following Peckham's decision, the *San Jose Mercury* suggested: "It would be far better for Larry P, and for students of every race, if educators would drop IQ tests entirely and rely instead on empirical evidence. Put the Larry P's in a regular classroom and see if they can handle the work. The time to move them into special programmes is after they have demonstrated an inability to keep up with their classmates."

John Douglas

## Dispute over Dow Chemicals' theory of dioxin traces

A Dow Chemical Company report on the toxic chlorinated dibenzodioxins, released in November 1978, has been severely criticised by Professor Christopher Rappe of the University of Umea, Sweden. He says that the methodology used by Dow is poor, rendering some of the results questionable; and furthermore, the conclusions — that dioxins are ubiquitous and a natural consequence of combustion processes — are far from proven. They are not borne out by results from Rappe's own laboratory.

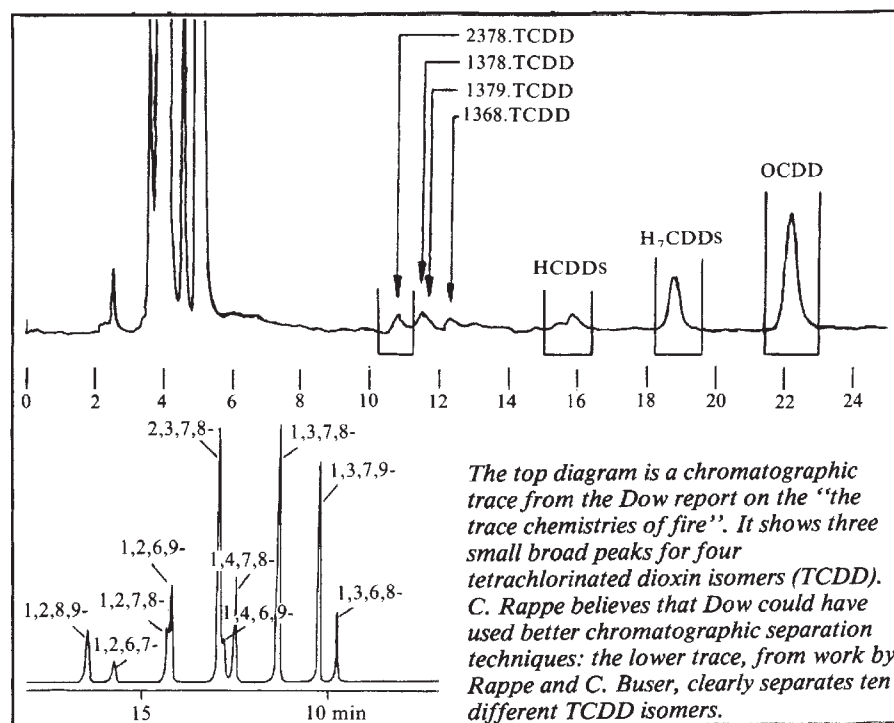
The report, on "The trace chemistries of fire", was prepared for the State of Michigan's Department of Natural Resources (DNR), after Dow found that fish taken from the Tittabawassee River contained measurable amounts of chlorinated dioxins and polychlorinated biphenyls. Effluent from Dow's huge Michigan complex is discharged directly into the river and, not unnaturally, the company was assumed to be the source of the dioxins. Dow now dispute this charge: their report claims that dioxins are produced in many combustion processes, and are widespread. It has not convinced the DNR, however, which is insisting on additional measurements. Dr Robert Bumb, Director of Research at Dow's Michigan complex, believes that such additional tests would not only be expensive, but of little value; he says that other scientists are now confirming Dow's discovery.

Professor Rappe told *Nature*, however, that he disputes many of Dow's findings. Their discovery that dioxins are produced

in commercial incinerators is not new, two independent European groups having reported earlier that the fly ash of municipal incinerators contains polychlorinated dibenzofurans. It was Dow's subsequent 'discovery' that chlorinated dioxins were present in the ash collected from other combustion processes which led them to develop their theory on the 'trace chemistries of fire'. Dow claim that dioxins are also present in ash collected from chemical tar burners, fossil-fuelled power plants, the 'mufflers' of automobiles and trucks, household chimneys, cigarettes and even charcoal-broiled steaks. There had to be a common factor to explain the wide occurrence and Dow developed the theory of the trace chemistries of fire — defined as 'numerous chemical reactions occurring during combustion at very low concentrations, parts per million and lower'. Yields from these reactions are very low, of the order of  $10^{-9}$  per cent.

The company attributes the formation of chlorinated dioxins to the presence of dioxin building blocks, which would include chlorine and chlorinated aliphatic and aromatic hydrocarbons. Metals present may act as catalysts "in a sea of chemical reactivity including pyrolysis, oxidation, reduction and acidolysis". In similar poetic vein, the report adds that in this sea, "ions, electrons, free radicals, free atoms and molecules form, combine and decompose". Chlorinated dioxins, Dow suggests, must be formed in this process.

Not necessarily, says Rappe. His results, those of colleague Dr Hans Rudolf Buser, and of Dr Hans Paul Bosshardt of



The top diagram is a chromatographic trace from the Dow report on the "the trace chemistries of fire". It shows three small broad peaks for four tetrachlorinated dioxin isomers (TCDD). C. Rappe believes that Dow could have used better chromatographic separation techniques: the lower trace, from work by Rappe and C. Buser, clearly separates ten different TCDD isomers.



the Swiss Federal Research Station, all suggest that the dioxin precursors are not so nebulous. Rappe considers that chlorinated phenols and chlorinated diphenyl ethers are the main precursors in municipal incinerators. When these two groups of compounds are heated under laboratory conditions, Rappe has found that they produce isomers of chlorinated dioxins in similar proportions to those seen in commercial incinerators. Although Rappe forwarded his results to Dow, it appears either that they were not interested or that they chose to ignore his findings, for there are no analyses for these precursors in the Dow report.

The chromatographic separation techniques used by Dow to identify dioxin isomers are poor, according to Rappe, and their samples should be reanalysed using another method (see diagram on p619). In Rappe's opinion, Dow have overestimated the quantity of the dioxin isomer 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) which would be present in the fly ash of municipal incinerators. This isomer is many times more toxic than any of the other chlorinated dioxin isomers studied so far, and Rappe says that it

represents only 1-3% of the tetrachlorinated dioxin isomers in European incinerator fly ash. Dow, on the other hand, report that in some cases 2,3,7,8-TCDD was the major tetrachlorinated isomer. Rappe suggests that this dramatic difference between his and Dow's results could be explained by the fact that Dow analysed samples from one of their own incinerators. Dow incinerates residue from the reactor used to make 2,4,5-trichlorophenol, the precursor of the herbicide 2,4,5-T. As the incineration of 2,4,5-trichlorophenol is known to produce 2,3,7,8-TCDD, Rappe suggests that the chlorinated phenol may have already been present in the reactor.

Rappe and his colleagues have so far prepared and identified 31 of the 75 chlorinated dioxin isomers which are considered to be theoretically possible. Although Dow used a far fewer (and unstated) number in its study, they believe that they identified enough isomers to make the report valid. In addition, Bumb points out that another Dow scientist, Dr D E Townsend has verified the trace chemistries theory from thermodynamic principles. Bumb told Nature that

Townsend, in an unpublished report, finds a striking correlation between observed and predicted values for the dioxin isomers. Rappe disputes one of Townsend's assumptions (a constant ratio between dioxin isomers with different numbers of chlorine atoms): "consequently his basic theory is wrong".

Dow appears to be unaffected by the scepticism of scientists in Europe and the US. Bumb claims that "results which depart from traditional and commonly-held beliefs routinely provoke scepticism". And he remains confident in Dow's results and the company's conclusions.

Bumb and Rappe will have a further opportunity to put their respective cases in November, when both will present evidence in hearings organised by the US Environmental Protection Agency. The EPA is to consider its ban on the herbicide 2,4,5-T, which it has recently extended, from a restriction to forestry only, to a total ban covering all known uses of the herbicide in the US. The danger of 2,4,5-T lies in its contamination with small amounts of the dioxin 2,3,7,8-TCDD, one of the subjects of contention in the Dow report.

Alastair Hay

## Israeli universities face financial crisis

ISRAEL'S seven institutions of higher learning have successfully resisted a recent attempt by the Knesset's Finance Committee to cut £8 million from their budgets.

A threat to double tuition fees or even close down campuses was enough to spur Prime Minister Menahem Begin to intervene. He successfully asked the committee to reverse its decision.

University officials are naturally relieved, but they are still fearful of future developments. Their mood was expressed by a top academic administrator who said that they "had won the battle and yet might go on to lose the war".

This is because the Knesset members who reluctantly agreed to restore the £8 million and many other influential Israelis apparently believe that the universities and academic research centres are characterised by luxury and waste, which means that they can more readily absorb budget cuts than can other institutions supported by public funds. And such cuts are inevitable if the rampaging, nearly three-digit inflation now plaguing Israel is to be brought under control.

The institutions of higher learning certainly helped to create their image in what Weizmann Institute President Michael Sela some years ago called "the Herodian era of construction", when universities, egged on by donors anxious to have their names associated with imposing edifices, seemed intent on surpassing one another in square metres of marble and concrete.

Yet there are also other, perhaps more

significant factors at work. To some extent, as in the West generally, science and scientific institutions are held responsible in Israel for almost all the ills of modern society. In addition, parliamentarians concerned about the problems of the underprivileged and poorly educated sections of Israeli society are ready to divert scarce funds from universities and research centres to nursery schools, primary schools and secondary schools.

In any case, even before their latest tangle with the Knesset's Finance Committee, local institutions of higher learning were already suffering from a severe decline in government support. In the period between 1972 and 1978, when the national budget grew in real terms by 30%, funds allocated to higher education declined in real terms by 20%.

Making things even more difficult for the institutions is the erratic manner in which government allocations are dispensed. Money does not come in regularly week by week or month by month; instead most of it tends to arrive towards the end of the fiscal year.

Yet the universities are prohibited by law from withholding wages, and if they don't pay for their supplies, the supplies stop. This forces them to take high interest loans in order to bridge the gap between current expenditure and eventual government grants. University spokesmen claim that economy measures have already gone past the stage where fat was being trimmed and now are impairing their ability to operate properly. According to Tel Aviv University President Haim Ben-Shahar, "libraries

and laboratories are no longer up to date. Journals containing important current research developments are sometimes impossible to acquire. Our scientists are forced to work with obsolete equipment, which, of course, puts them at a disadvantage in comparison to their colleagues abroad".

A research chemist at another institution told of a case in point. For some years, he said, the people in his department have been seeking funds to purchase a gas chromatograph mass spectrometer. At first they could go on with experiments by constantly tinkering with their old mass spectrometer, purchased in the early 1960s. But now some lines of research have had to be dropped as it is impossible to obtain relevant and significant results without the newer instrument.

Financial problems have severely limited the hiring of new staff, leaving institutions of higher learning with a disproportionate number of aging, tenured men and women. This news about job prospects has reached university students, and it undoubtedly has something to do with declining enrolments in the natural sciences.

Chemistry faculties are particularly hard hit, with registration down by anywhere from a quarter to three-quarters and some institutions now boasting more teaching staff than students. The same applies to a lesser extent to other science faculties. Students are now choosing short courses promising good employment prospects and the possibility of high financial gain.

Nechemia Meyers

# Public spending cuts threaten UK universities

THE UK government is on the brink of a decision which could be disastrous for the country's universities, according to Sir Alec Merrison, Chairman of the Committee of Vice-Chancellors and Principals. Proposed cuts in funds would almost certainly mean the closure of some departments and even whole universities, he told a press conference last week, and universities would not be able to educate well-qualified home students whose numbers are likely to continue increasing over the next few years.

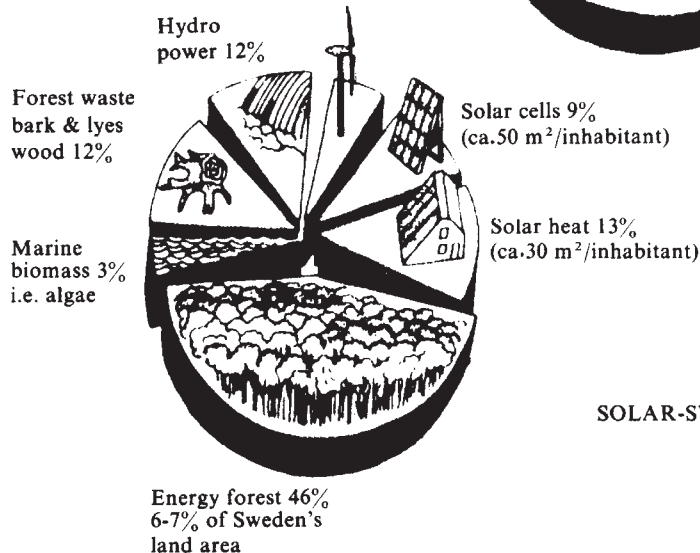
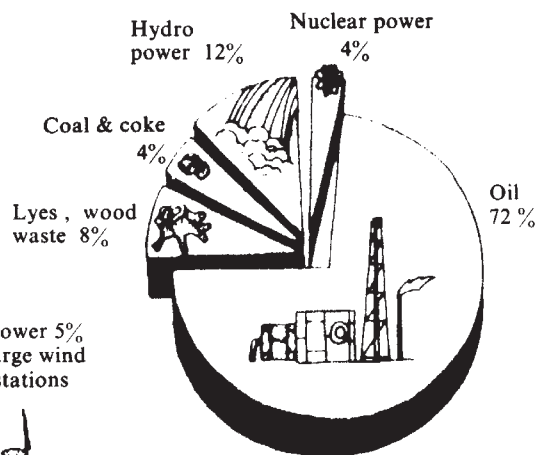
The CVCP was responding to the government's intention — outlined in a letter from the University Grants Committee to all universities last week — to hold the universities' recurrent grant at the 1979-80 level for several years and remove all UK government support for overseas students embarking on their studies on or after the 1980-81 academic year.

Cutting out funds for overseas students would result in a 13% fall in university income by 1983-84. The money could only be recovered if large numbers of overseas students were capable of paying their own fee — estimated at £3,500 each. This is highly unlikely, says the CVCP in a letter to Mark Carlisle, the Education Secretary. Overseas students would have to pay many times more to study in the UK than in other western countries and would therefore go elsewhere. British universities would cease to compete internationally. Some universities would be hit very badly, for example, the University of Manchester Institute of Science and Technology, a third of whose students come from overseas.

The vice-chancellors are concerned that research as well as teaching is bound to suffer. In looking for economies, universities would tend first to cut spending on research equipment. "The whole question of research is considerably at risk", Sir Alec said.

A further problem for universities is uncertainty about the 1979-80 level of funding which is to be used as the base for the recurrent grant until 1983-84. Salaries for university staff have yet to be agreed for the year ending July 1979 and the government has given no assurance that it will meet any increases. The CVCP is also concerned that pegging their future funds at 1979-80 levels will not take account of inflation and pay increases to come in the next few years. Many universities are already having difficulties existing on their funds. Sir Alec's own university, Bristol, ran up a deficit of £500,000 in an income of £25 million last year. "I'd be very surprised", he said "if there aren't some institutions that have had to go to the bank already." □

OIL 1978



SOLAR-SWEDEN 2015?

Changing the energy equation: how Sweden's forecasters see the future

## Sweden warms to biomass power

INDEPENDENCE from oil as a source of energy was the basis of a Swedish contribution to a seminar on Technological Forecasting Methodologies recently held by the UN Economic Commission for Europe (ECE). The authors Thomas B Johansson of Lund University, and Peter Steen of the Swedish Research Institute for National Defence, suggest that 61% of the nation's energy could be provided by biomass. Six to seven percent of the country's land area, they propose, would be devoted to energy forests, estimated to yield 46% of the country's needs; forest waste and wood lyes (pulp effluent) would contribute 12% and marine biomass 3%. Other contributors would be solar heat (13%), conversion through solar cells (9%), wind, and hydro-power (12% — the same as the current figure).

No mention however is made of methane from the conversion of urban waste, a resource already used in some other north European countries. The figures are contrasted with those of the present system, which relies on oil for 72% of the country's needs, hydropower for 12%, woodwaste 8%, coal and coke and nuclear 4%. Neither coke nor nuclear appears in the 2015 forecast, which is said to allow for "moderately increased efficiency" and "twice the production of goods and services compared with today".

The authors have assumed that "the energy system should be fully implemented in the year 2015", and have calculated backwards the necessary rate of expansion. This gives 50% implementation from renewable resources by the year 2000. The rate they estimate is less than that estimated by the Swedish National Board for Energy Source Development. At the same time the cost of the proposed system is "not in conflict with production levels assumed for the whole economy" while it is based on a continued increase in labour productivity.

But there are difficulties. For example, there are to be major political and economic issues with regard to land-use policy and the capital market, since the proposals would require more land area and are more capital extensive than the present energy system. However, Sweden is already very advanced in research on energy plantations, making use of selected strains of various tree species grown on a short rotation system and giving yields comparable to those found in the tropics.

Two German papers at the seminar, however, estimated that solar energy would contribute only 5% of all energy by the year 2000. They paid little attention to the indirect uses of solar energy through biomass conversion or the better use of wood and wood-products. However, several other ECE meetings have been



devoted to the energy aspects of the forest industries. At one it was indicated that the energy input of the industry itself to pulp and paper manufacture ranged from 5-35% in different countries, with a very much lower figure (5% only) for the input to the mechanical processing side. Sweden, again, is a leader in examining how these figures could be improved by more efficient use of forest waste: one recent estimate gives 50 million m<sup>3</sup> of residues consumable

as fuel if not partially recovered for other processing, for every 60 million m<sup>3</sup> of commercial round wood produced annually.

Again, in Finland it is estimated that small sized wood, stump/root systems and other forest waste could provide 3.6 million tons oil equivalent a year, with another 0.8 mtoe depending on the market for certain hardwood products. Timber industries, faced with rapidly rising energy

costs from conventional sources, are examining new ways of using their own waste products, either by direct combustion or through conversion into gas or liquid fuels. An advantage is that wood is not carcinogenic and is low in both nitrogen and sulphur. According to one ECE report, forest product industries can and should become energy independent soon, later supplying surplus biomass to other users.

Peter Collins

## Poland calls for wider research cooperation . . .

International cooperation at every level — from pure academic research to industrial design and implementation — is essential if a country's research potential is to develop to its full capacity. So Jan Mazurkiewicz, Director of 'Export and Economic Relations with Abroad' of the Polish Ministry of the Machine Industry told *Nature* recently. He was summarising the significance of "Poland's Technology, 1979" a series of seminars and technical exhibitions held last week in London, Manchester, Birmingham, and Coventry.

The close integration of research with industry is a basic feature of Polish planning. It was perhaps less familiar to some of the British participants, who seemed a little at a loss as to whether to expect learned discourses or sales promotion talks. For the event, sponsored on the British side by the Chambers of Commerce of the host cities, was the reciprocal or a similar British week in Warsaw last autumn, and represents one of the more positive results of Poland's large trade gap with Britain.

If trade between the two countries is to increase — and the state of the Polish economy makes this highly desirable — some new form of cooperation must be found. Recently, joint research and development projects, culminating in joint production for a third market, has increasingly been proposed on both sides as an attractive solution.

Any viable proposal for joint projects presupposes, that both sides know what the other has to offer. "A symposium means the meeting of people with like interests", Mr Mazurkiewicz explained. "So we chose our team very carefully — a couple of academicians, a test pilot, two plant engineers, an administrator, and so on. We looked for a good variety, for people with open minds, with whom there could be a point of contact."

The subjects, which ranged from medical electronics to machine tools and from computer software to agricultural aviation, were selected by the Polish side as fields where they felt they had something valuable to offer. Inevitably, in the discussion of any cooperation projects, sooner or later the problem of strategic military embargoes crept in. These, however, were peripheral, and in one instance, Jacek Szporko of "Unitra-

Elektron" joked that the strategic embargo of the early 1960s had indirectly led to Poland's present expertise in semiconductor technology.

The close links between Polish research and industry meant that the Polish team could include lecturers of considerable international standing. Andrzej Radziminski (software and computer services) is a member of the Advisory Committee for Informatics Projects of the International Bureau for Informatics Dr Tadeusz Zak, who lectured on the Polish technological equipment industry, is a member of the Comecon Plenipotentiary Committee for Corrosion Problems, and Dr Halina Leszczynska (industrial sulphur processing) was closely involved in the development of granulated sulphur. This is free of the health and environmental hazards that had been closing more and more ports throughout the world to sulphur in its traditional powder form.

Dr Leszczynska is a member of the Committee of Chemical Sciences of the Polish Academy of Sciences, and, as such, represented a field which, she said, is always in the "top three places of research investment", due to its "importance in the

national economy". She was particularly optimistic about the outcome of the week. "There are already a lot of people doing joint research at post-graduate level", she said. "What this week has done is to provide new direct contacts at the development level."

Several members of the Polish team, in informal discussions, suggested that joint industrial and development projects should ideally be based on truly joint research, rather than bringing together the results of research at a late stage.

Although the symposium, was slanted rather towards the industrial end of the research and development spectrum, the lectures from time to time revealed interesting sidelights on Polish research structure which would undoubtedly have to be taken into account in the planning of any joint research. Who would have guessed — until test pilot Wieslaw Mercik pointed it out — that the Warsaw Aviation Institute does no work at all on whole-body helicopter design, but is simply responsible for testing the aerodynamic and performance characteristics of designs produced in the factory design offices?

Vera Rich

## . . . while the East Germans must wait for a trade agreement first

THE German Democratic Republic, which celebrated its 30th anniversary recently, is a country where, according to Dr Lutz Buschendorf, First Secretary (Science and Technology) of the GDR Embassy in London, every single worker is to some extent involved in science. Unfortunately, he said, so far scientific cooperation between the UK and the GDR has not developed to the extent one might have hoped. The reason, from the East German point of view, is the "unwillingness" of the UK to sign a cooperation agreement in which science and technology would not be linked to trade.

At present, technological cooperation comes under the Joint Agreement, originally drawn up in 1973, in which the British side comes under the aegis of the Department of Trade. This is the standard situation for all UK cooperation deals with Eastern Europe. The involvement with trade has a two-fold basis. Firstly, from the

beginning, the UK felt that if the agreements were to bring genuinely mutual benefits, then some kind of counter-trade would be needed to off-set a predominantly one way, West to East flow of technology. Secondly, the administration of the agreements by the Department of Trade (via a special technology unit) is a result of the way the ministry of technology was dismantled.

To the East Germans, however, the link with trade constitutes a major psychological block. When the then UK Secretary of State for Trade, Edmund Dell, visited the GDR some three years ago, he stressed that under existing economic conditions the trade turn-over between the UK and the GDR could well be doubled. Some Germans seem to have taken this as a direct challenge to their thesis that increased scientific and technological cooperation should pre-date any major expansion of trade. "We want to make



cooperation more long-term", Dr Buschendorf explained. "Under the present agreement it is difficult to get the right partners for scientific cooperation".

From the German point of view, the situation was somewhat improved by the signing, last May, of an agreement for the exchange of scientists, between the Royal Society and the GDR Academy of Sciences. Otherwise, however, they remain intransigent that what they need is a separate cooperation for science. They do not even seem willing to discuss possible areas of cooperation until such an agreement is signed — even to suggest subjects, Dr Buschendorf felt, would be "premature". It is known, however, that the British side has put forward a number of concrete proposals for joint seminars and visits, in order to bring together specialists who could then discuss feasible cooperation programmes. Subjects in which Germans have indicated particular interest include industrial hydraulics and corrosion protection. So far, however, there has been no firm response.

The insistence of the GDR on keeping science and trade firmly separated seems a little incongruous in the light of its own science policy. This, according to the official hand-outs for the current anniversary, is based on "a close and desired connection" between research and "the tasks set in the national economy". Innovation and implementation are the keynotes, and workers at all levels are encouraged to suggest further possible improvements. There are close funding links between industry and basic research. The production of university graduates is also a part of state planning, and each student is provided with his or her future job at least a year before graduation.

Given an economic structure, where "all the planning of scientific and technological work is based on the requirements put on the development of the national economy", it is difficult to understand the Germans' reluctance at least to explore what can be done under the existing agreement. Many of the fields which have priority in the GDR research programme — environment, water pollution, energy and raw materials, computers, could provide considerable scope for joint work with the UK or other western countries.

Perhaps some part of the explanation may lie in a report produced last spring by the East Berlin Institute for Politics and Economics. The Institute team, it appears, was apprehensive that injudicious importing of licenses could harm indigenous research and development by turning the national research potential into a mere mechanism for the adaptation of foreign thinking.

So far, however, no negotiators have made any reference to this point of view, but, on the thirtieth anniversary of GDR statehood, have raised yet again their plea for a separate scientific agreement.

Vera Rich

## Soviet scientist proposes Siberian sites for nuclear power stations

Soviet nuclear power stations should be sited in remote regions of Siberia and the Far North, according to Academician Nikolai Dollezhal, a leading nuclear scientist, and Yuri Koryakin, a Doctor of Economic Sciences. Moreover, such power stations should take the form of large "nuclear energy complexes", each consisting of a number of power stations of "several tens of millions of kW capacity" together with fuel reprocessing plants, and possibly facilities for utilising nuclear byproducts.

This proposal, which the authors themselves admit is "radical", appeared in the September issue of *Kommunist*, the leading Party theoretical journal. Starting out apparently as an encomium of Soviet nuclear progress, the tone gradually becomes less optimistic, and, after outlining the setbacks and delays of the fast breeder programme, the authors openly tackle the environmental problems, hitherto dismissed by Soviet publicists as scare-mongering by capitalist oil interests. Reprocessing risks are particularly stressed, and bolstered by the assertion that basic reprocessing costs are "significantly" higher than originally estimated.

Still more costly, they imply, is the loss in the productive land and water resources. Some 60% of the Soviet population live west of the Volga river/Volga-Baltic canal

line. It is here, in the main area of industrial and agricultural production that it is currently proposed to site nuclear power stations. The annual water requirements of nuclear and thermal power-stations already exceed 100 km<sup>3</sup>, some 2 km<sup>3</sup> of which are "irreversibly" lost. To accommodate this water, by the end of the century, if current construction patterns continue, agricultural land sufficient to provide bread for "several million people" will have been sacrificed.

Dollezhal and Koryakin would not, they stress, cast any doubt on the "historical necessity" of nuclear energy. Without nuclear energy, they affirm "it is impossible to construct the energy basis of developed socialism".

Giant energy complexes in Siberia, they claim, might well prove to be more efficient as regards construction, operation, and the "utilisation of labour reserves". Environmental hazards would be removed to a safe distance, and those associated with the transport of fuel from reactor to reprocessing plant eliminated completely. Finally — and this might well be the point that would win over the decision makers — the proposed nuclear complexes could be integrated into the Soviet Union's most prestigious civil engineering project — the diversion of the Siberian rivers to replenish the arid south.

Vera Rich

## Physicist flown home to face charges

Franco Piperno, the Italian physicist who had asked for political asylum in France, was extradited to Italy last week. The extradition order was given a week in advance of its scheduled hearing to avoid demonstrations and rescue attempts.

Piperno, aged 36, was escorted from Sante prison at 7am to Le Bourget Airport where an Italian Air force plane took him to the small military airfield, Pratica di Mare, north of Rome. He was whisked to solitary confinement in the political wing of the modern Revibbia jail in Rome.

Students in Rome set fire to cars and buses to protest at Piperno's extradition from France and police fired volleys of tear gas in unsuccessful attempts to disperse the demonstrators.

Forty five counts of extradition ranging from stealing car license plates to armed insurrection and including offences against the highway code were rejected by the French Court of Appeal (as was a first extradition request) on the grounds that they were political in character and violated the 1870 extradition treaty between the two countries.

But the court released Piperno to the Italian authorities to answer charges that he had sheltered two men in whose homes Italian police had found guns allegedly

used in the abduction of Aldo Moro. Moro, one of Italy's political elder statesmen, was kidnapped and later killed by the Red Brigade guerrilla group in Rome after their attempts to exchange him for jailed comrades had failed.

Piperno had denied involvement in the Moro affair. His statement at the extradition hearing was: "Perhaps it is better this way. At least I will be able to confront quickly that which in Italy is called justice and to demonstrate that I was not involved in the Moro kidnapping and assassination."

In Paris, where Piperno had received widespread support, French commentators expressed dismay and anger at the extradition. Francois Mitterand, leader of the Socialist opposition, declared that the Socialist Party "deplores the fact that French judges have yielded to governmental pressures". And Philippe Boucher, writing in *Le Monde*, said: "It is plainly unreasonable to believe that Italy had no political interest in brandishing Piperno as proof of their determination to combat terrorism. No one can seriously maintain the contrary . . . The (judicial) method in this case is contestable, indeed unseemly. It is furthermore dangerous."

□

# Who shall inherit the seeds of the earth?

"Seeds of the Earth", the study recently released by the London-based International Coalition for Development Action (ICDA), raised several issues of international concern. The study's author P R Mooney predicts that the Green Revolution is now moving into its second phase — a dangerous phase in which the responsibility of plant breeding is largely being left to the large private multinational companies. The study contends that new legislation in the developed countries which gives patent-equivalent protection to producers of new varieties of plants is helping the multinational corporations to acquire dominance over the seed market.

These trends are dangerous, according to the ICDA study, because of the gradual erosion of the world's plant genetic resources that is taking place all across the Third World. This erosion process has been accelerated in the last decade by the Green Revolution which has helped to bring vast areas under cultivation of monocultures. Deforestation is another aggravating factor. The Vavilov centres of genetic diversity (see map) — the geographical regions from which plant breeders draw a large part of their germplasm — are mainly located in the Third World. This germplasm is vital to the future food supply systems of mankind, as it is needed by plant breeders for further improvement of crop yields and incorporation of other desirable characteristics such as disease and pest resistance.

'Seeds of the Earth' points out that private corporate interests which are entering the seeds business are building up private gene banks to which access is limited only to the companies' plant breeders. This situation is particularly ironical for Third World plant breeders as Third World countries are the original suppliers of these plant resources.

Little is known about private germplasm collections, but the study does claim that "in some crops a single enterprise dominates total world germplasm holdings". "The FAC reports", says the study, "that one company, United Brands (formerly United Fruit), has about two-thirds of the world's banana germplasm in storage". Some public gene banks have had difficulty in obtaining information from private companies about the quantity of type of genetic material they hold.

At the international level, germplasm conservation is the responsibility of the International Board for Plant Genetic Resources which is based in Rome within the Food and Agriculture Organisation of the United Nations. The board co-ordinates the regional germplasm collection work that is being undertaken by the eight existing international crop research stations. There are also some 60 nationally-controlled gene banks. The

germplasm collection work is massive and the board's activities remain clearly underfunded. There is little germplasm collection work being undertaken by Third World governments themselves, whose plant heritage is immediately threatened.

The ICDA study, therefore, recommends the creation of an annual emergency budget of US \$100 million for the collection and storage of genetic material and creation of biosphere reserves. But equally important, the study points out, is the step that the UN should take to declare plants as "resources of common heritage to all peoples". There should be no form of legislation that allows exclusive control over plants. Secondly, the study recommends that the genetic material collected should be stored in the developing country from where it is collected. Most of the germplasm being collected at the moment is stored in the developed countries. If this trend continues, the Third World, the original supplier of this vital resource, could soon find itself dependent on the developed countries for the basic material required for its own plant breeding programmes.

The immediate target of attack of the ICDA study is the new legislation that is pending in several developed countries. This legislation provides patent-like protection to new plant varieties. It is the opportunities provided by this type of legislation — often called legislation for "plant breeders' rights" — that has encouraged the entry of big corporate interests, much to the detriment of the small breeders who are now being squeezed out of the market or being bought up. The ICDA study, in fact, claims to have been born out of the movement that farmers in Saskatchewan province of Canada have launched against the pending Canadian bill on plant breeders' rights.

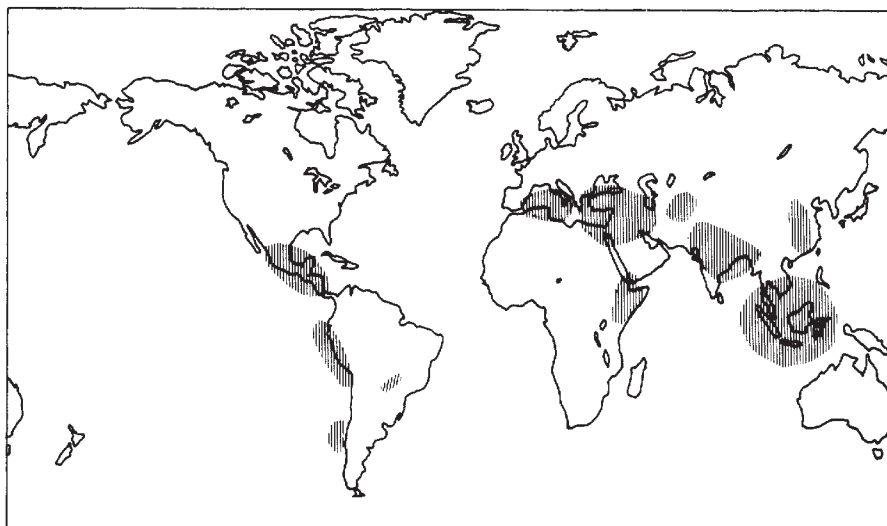
Unfortunately, the study fails to provide adequate evidence for some of the charges

it makes. For instance, the argument that the entry of agrochemical companies into the seeds business is "a way of assuring that one product line needs the other" remains at the moment a mere hypothesis. Patent protection for many important pesticides that were developed in the 1960s and earlier has already expired or is about to expire. This has raised problems for many companies which are now taking various steps to protect their interests either by diversifying into related areas or by somehow ensuring that their sales are not affected by expiry of patents. As agrochemical companies already have considerable scientific and technical expertise in various branches of agricultural sciences and a good marketing network that reaches right out to the farmers' fields, the seeds business may just be the ideal area in which to diversify.

Similarly, the World Intellectual Property Organisation which is responsible for the International Union for the Protection of New Varieties of Plants (UPOV) believes that the fears raised about the effects of the PBR legislation are exaggerated. An article of the International Convention for the Protection of New Varieties of Plants clearly states that no authorisation is needed to use a protected variety to produce yet another variety — except in the case where "the repeated use of the new variety is necessary for the commercial production of another variety," as in the case of hybrid varieties. Thus, the PBR legislation should not in itself prove to be a major impediment for further plant breeding work.

But despite its deficiencies, there is no doubt that the ICDA study raises several issues of major concern to mankind, and these issues needed to be fully debated to protect public interest, both in the developed and the developing countries.

Anil Agarwal



*The Vavilov centres of genetic diversity: the Mediterranean, the Near East, Afghanistan, Indo-Burma, Malaysia-Java, China, Guatemala-Mexico, the Peruvian Andes and Ethiopia.*



## news in brief

**Birmingham University smallpox case starts:** Legal action against Birmingham University under Section 2 of the UK 1974 Health and Safety at Work Act began this week in Birmingham. The university is being prosecuted by the Health and Safety Executive for failing in its obligation to "ensure as far as reasonably practical" safe working conditions in the east wing of the University Hospital. The release of smallpox pathogens from a P3 containment facility late in 1978 resulted in the death of a laboratory worker and closure of the facility. Under the terms of the act, the university could face a maximum fine of £1,000.

UK experts in occupational health regard the case as an important precedent that goes beyond the guilt or innocence of the university. It is the first prosecution brought against a university since the act was passed in 1974. Sheila McKechnie, health and safety officer of the Association of Scientific, Technical, and Managerial Staffs, said the case had given impetus to the campaign for laboratory safety. "The universities have been hiding behind a lot of 'frontiers of science' rubbish. They have been arrogant and have refused to acknowledge their obligations under the 1974 Act. The smallpox case has raised everyone's awareness of the extent of health hazards involved in university research", she said.

**US studies identify formaldehyde as carcinogenic:** Laboratory rats exposed to formaldehyde vapour have been shown to develop nasal cancer, according to tests submitted to the Consumer Product Safety Commission by the Formaldehyde Institute recently. A spokesman for the institute said that in the course of a two-year study, four rats out of a total of 720 tested developed tumours visible to the naked eye after 16 months, three after exposure to formaldehyde exposure at levels of 15 parts per million, and one at a dose level of six parts per million. Under existing standards promulgated by the Occupational Safety and Health Administration, worker exposure must be kept below three parts per million over an eight-hour period, or an average of five parts per million over 15 minutes. Mr John Clary, chairman of the institute's medical committee and a toxicologist with Celanese Corporation, said that the results were "significant".

**ARMS to lobby parliament:** ARMS, the Association for Researchers in Medical Science, are holding a day of parliamentary activity on 6 November. Members and supporters will assemble outside the Department of Education and Science to present a petition to the Secretary of State for Education and Science demanding "immediate action to implement a proper career structure for research scientists in biomedical research in UK universities and medical schools". A meeting is then planned with a number of MPs to discuss the position of medical researchers in the UK. An ARMS spokesman called the action "perhaps the last chance for researchers on short term contracts to discuss with MPs what is wrong with the current system of funding in the UK".

**Chinese Minister of Education in whirlwind tour of UK:** Mr Jiang Nanxiang, Minister of Education of the People's Republic of China, visited the UK from 28 September to 7 October to discuss scientific exchanges and to see British educational institutions. Mr Jiang, who had been relieved of his duties as principal of Peking University during the Cultural Revolution, was appointed Minister of Education earlier this year. He is said to be a believer in "quality education". The one-week tour consisted of visits to 18 UK educational and scientific institutions including middle schools, polytechnics and the Clarendon and Cavendish laboratories at Oxford and Cambridge. The Chinese delegation expressed particular interest in the Open University and its sophisticated system of correspondence courses, television programmes and regional tutorial centres. According to an OU

spokesperson, the minister expressed interest in the regional centres, home experiment kits, and asked whether OU degrees were useful for "advancement and promotion". The university is designed to make university education more readily available to people from working class backgrounds but the Chinese minister expressed "little interest" in this aspect of the university's work, an OU official said.

**India achieves breakthrough in fish culture:** India's fish production may be revolutionised by a new composite fish culture technique developed by the Central Inland Fisheries Institute, (CIFRI), Barrackpore, West Bengal. The new culture technique uses a compatible fish combination of indigenous major carps and fast growing carps, and scientists testing this found yields as high as 6,500 kg fish per hectare per annum could be produced in some regions, although the project target had been only 3,000 kg fish per hectare per annum. This culture can be integrated with pig and duck culture, reducing the operational costs and producing 4,500 kg fish per hectare per annum and 1,800 duck eggs. Pig and duck excreta supply nutrients to the water, maintaining a high level of production. CIFRI's director, Dr V G Jhingran, estimates that if all India's present culturable waters are put to composite fish culture, average fish production will rise from 600 to 3,000 kg fish per hectare per annum. — Zaka Imam, New Delhi

**Most blindness can be prevented:** A meeting of an international group of experts on blindness has produced a report claiming that the incidence of blindness in the world can be halved quickly by simple and effective preventive measures. According to the report, most of the estimated 30-40 million cases of blindness are due to infection, malnutrition and cataracts. Visual impairment is 10-40 times more prevalent in certain areas of developing countries than in industrialised countries. The main obstacles to initiating a prevention campaign are a lack of national commitment, inadequate administrative infrastructure and a lack of money. The report also urges an avoidance of "the misunderstanding that equates the prevention of blindness with the availability of ophthalmologists". The meeting was sponsored by the F.I. Proctor Foundation in San Francisco, The International Eye Foundation, and the World Health Organisation. *Guidelines for Programmes for the Prevention of Blindness.* World Health Organisation, Geneva, 1979. 47pp. Sw Fr 10.

**IAEA reports 50% increase in new nuclear plants:** The International Atomic Energy Agency in Vienna, in its annual survey of nuclear energy, reports that 15,000MW of new nuclear plants were installed in 1978 compared to 10,000MW installed in 1977. Nuclear power now accounts for 110,000MW or 5.8% of the world's total electrical generating capacity with 227 nuclear power stations operating in 21 of the agency's member states. The agency defends the safety record of nuclear power, citing "twenty years of commercial nuclear power generation without a single radiation-induced death or a serious radiation induced injury at any nuclear power plant".

**EEC to spend £70 million on energy research:** The Council of the European Communities is to spend about £70 million on a second four-year programme into energy research and development. The money will be allocated on the basis of shared-cost contracts over the four-year period 1979-83. Research organisations, industrial companies and universities will be eligible to apply and the first formal call for applications has been made by the EEC. Applications will be screened by an advisory committee which will make recommendations to the commission. The breakdown of the allocation is: solar energy, £30.7m; energy conservation, £18m; geothermal energy, £12m; hydrogen energy production, £5.3m; and energy strategy studies, £4m.





## **'There is a frustrating logical gap between the assertion that recombinant DNA poses potential risks . . . and the containment levels imposed by current guidelines'**

**Francis Rolleston** proposes a formula to bridge the safety gap in recombinant DNA research and concludes that any approach which falls short calculation of the risks is necessarily misleading.

THE UK Genetic Manipulation Advisory Group (GMAG) has proposed an important new way to assess the relative possible risks of recombinant DNA experiments (9 November 1978, page 104), but it addresses only the assessment of risk, without establishing the necessary correlation between risk and the containment required to reduce the risk to acceptable levels. Here I will suggest a framework for discussion based on this essential correlation. This framework also offers a clarification of the roles of the scientists and the public in setting safety standards, and establishes a foundation for communication between them.

All current guidelines make quantitative statements; they are safety standards which state, in effect, that the inherent risks can be reduced to acceptable levels of public risk by the imposition of defined safety procedures. Yet these quantitative statements cannot be defended either quantitatively or logically. The guidelines therefore do not provide a foundation for rational interchange either among scientists themselves, or—more important—between scientists assessing risks and containment procedures and the public, who should be reviewing the assessments by the scientists and setting acceptable standards of public risk. There is a frustrating logical gap between the assertion that recombinant DNA poses potential risks on the one hand, and the containment levels that have been imposed by current guidelines on the other.

*The author is director of special programmes for the Medical Research Council of Canada, with responsibility for regulating recombinant DNA research.*

Logical guidelines must be both internally and externally consistent. Questions of internal consistency are illustrated by the option sometimes offered of a trade-off between physical and biological containment (do P2-EK1 and P1-EK2 offer similar containment?), or by the assignment of different experiments to different containment levels (are the same safety standards being achieved?). External consistency raises the question of whether the safety standards imposed for recombinant DNA are consistent with those required or accepted in other areas.

A conceptually simple framework that may meet these needs for consistency is offered by the formula for a straight line,  $R = aC + T$ . Here,  $R$  describes the risks inherent in cloning a certain gene in a certain host-vector system,  $C$  is the physical containment required, and  $t$  is a threshold level of acceptable public risk to which inherent risks ( $R$ ) must be reduced, if necessary, by physical containment ( $C$ ). In this general framework, the need for internal consistency is met if  $a$ , the slope, is equal to one. The value of  $T$  determines the external consistency of the containment requirements. The required distinction between the scientific and the public roles is achieved by recognising that the values of  $R$  and  $C$  are determined primarily in the scientific domain, whereas  $T$  should be set in the public domain. Both the numerical values and the explicit separation of the scientific and public roles in this equation allow—even force—the necessary interchange between scientists and the public.

In using this framework, the two variables  $R$  and  $C$  can be assessed in either

an absolute or a relative sense. The absolute approach requires interrelated units in which to express  $R$  and  $C$  and therefore  $T$ . In the alternative approach,  $R$  and  $C$  do not have units, but are expressed relative to reference points; declaration of a relationship between the two reference points will define  $T$ , since the slope  $a$  must, for internal consistency, equal 1. The absolute approach is more satisfying, but probably can only be used in areas such as radiation damage or automobile travel where risks can be clearly identified and assessed; the relative approach appears preferable where risks are conjectural.

Such a relative approach to values for  $R$  was developed by Brenner and applied by Rigby, and their manuscripts formed the basis of the paper by GMAG. Their reference point was a very hazardous possible experiment in which the pure gene for cholera toxin is cloned and expressed in wild *E. coli*. The risks of other experiments relative to this reference were expressed in terms of three separate factors (damage, expression and access) which, multiplied together, gave values ranging from less than  $10^{-16}$  to 1; these values were stated by the authors to be overestimates of relative risk.

The relative approach can also be applied to values of  $C$ . The reference point might be open to bench experimentation (P1 containment). With respect to researchers inside the laboratory, it can be suggested that a P2 laboratory offers perhaps  $10^2$  greater protection, allowing for the fact that the relative safety of a containment cabinet is limited by HEPA filter standards, and that not all procedures are carried out in such cabinets. A P3 laboratory offers perhaps a further protection of  $10^2$  since here airflow requirements reduce persistence of aerosols and more procedures are carried out in containment cabinets; and a P4 facility represents  $10^6$  greater protection than P1, again considering investigators inside the laboratory. Estimates for people outside the facility, or for the environment, result in smaller relative protection figures, since the only barrier is the HEPA filter, although the absolute hazards for those who work outside the facility are evidently much less than for those inside.

These primary figures illustrate how values for  $R$  and  $C$  might be developed by the scientific community. If the figures given have any significance the range of values for  $R$  is much greater than that for  $C$ . Therefore, the value assigned to  $T$  by the public process will determine whether some possible experiments are too hazardous to be performed at even the highest levels of physical containment, or alternatively other experiments present such low hazards that physical containment measures are superfluous; or perhaps both. If, for example, the reference experiment of cloning the

cholera toxin gene in a wild type *E. coli* requires P4 containment—stated by the Council of the Royal Society Report (*Nature*, 15 February, page 509) to be too severe a level for *Vibrio cholerae*—then any experiment with values for  $R$  less than  $10^{-6}$  (the resulting value of  $T$ ) can be performed at P1 or less containment. From the above figures, it appears that this would include most experiments now requiring higher levels of containment, and it would, in large measure, incorporate the conclusions reached at Falmouth, Ascot and Wye.

A second conclusion from these tentative ranges of  $R$  and  $C$  is that the value of  $a$  is considerably greater than one in the original American, British and Canadian guidelines (which tended to spread the range of experiments across the

available physical containment levels) and even in the revised American and Canadian guidelines. Therefore, if it is claimed that the more hazardous experiments have been appropriately contained, then those with less risk have been over-contained; conversely, if the apparently less risky experiments have been set at suitable containment levels, then containment standards for those of greater hazard have not been strict enough. Extrapolation from current scientific opinion (as expressed at Wye, without as yet any voice to the contrary) indicates that, if one must choose between these two claims, the former would be more accurate.

This proposed framework rests on assigning numerical values to risks and containment. Such an approach has been

criticised (15 February, page 509 and 8 March, page 113) as being too imprecise because the risks are conjectural and probabilities of human error cannot yet be assessed. However, the current approach to guidelines makes just such judgements without admitting to them. A formal framework for setting safety standards which also segregates the scientific and public roles, while providing a means of communication, may focus attention on specifics as opposed to generalities, and require those who make pronouncements of doom or optimism to define their statements more accurately. □

*I thank Mark Pearson for a most useful discussion of the manuscript, and John Keyston whose comments in September 1977 on the need for interrelationships between risk and physical and biological containment I did not understand at the time.*

## MIT puts some polish on a tarnished image

The Massachusetts Institute of Technology has raised \$4 million to establish an ambitious College of Science, Technology and Society. Supporters say it will help give MIT-trained scientists and engineers “an education for the 21st century”; others claim it is an attempt to protect private industry from criticism of its use of science. **David Dickson reports**

WHEN Dr Paul Gray spoke to the press earlier this month following his election as the next president of the Massachusetts Institute of Technology, he placed particular emphasis on the steps which the institute is taking in response to public criticism of the social impacts of science and technology.

The current centrepiece of this face-lift is an ambitious plan to create a new College for Science, Technology and Society at MIT. Already \$4 million has been raised from outside to support the project; and a programme of research and undergraduate teaching has begun as a development phase, supported by a number of eminent faculty appointments (including the historian of science Thomas Kuhn).

There is nothing particularly unusual about a technological university starting a programme in this area. What distinguishes MIT's efforts, however, is partly the size and scope of its commitment; and partly the fact that the programme is less a direct product of concern about the social impact of science, than a second order “reaction to public reaction” about such impact, such as the growing debates over issues from nuclear safety to the carcinogenicity of food additives.

And this has provoked its own controversy. The architects of the course claim that it is “a way of matching the maturing desire of MIT to go in this direction with the maturing needs of the nation”; critics warn that the institute is being used to promote the strategies of private industry in politically charged debates over technological risks and the appropriate social response.

Despite outward appearances, MIT is no stranger to the humanities. Various moves have in the past been made to complement its more scientific and technical courses; the Lewis Report of 1949, for example, led to the setting up in the 1950s of both a school of humanities and social sciences, and the Sloan School of Management. More recently, there have been particular developments such as a Technology Studies Programme and a Technology and Culture Seminar.

The aim of the proposed college is to focus these various activities and give them a new prominence within the institute. The move stems largely from an initiative taken by Dr Jerome Wiesner, ex-science adviser to President Kennedy and current president of MIT, who set up a faculty task force in 1973 to advise on how these goals could best be achieved.

After consulting with numerous individuals inside and outside MIT, the task force recommended the setting up of an independent college, with its own faculty, research fellows and undergraduate teaching programme. Based on this report, the institute raised \$2.5 million in 1977 from three foundations, the Sloan Foundation, the Mellon Foundation and the Flora Hewlett Foundation; a further \$1 million was added this year from the Max Fleischmann Foundation for renovating an appropriate building, and a \$500,000 grant for a fellowship programme was made by the Exxon Foundation.

With this support under its belt, MIT has

already been able to make seven new tenured appointments, and introduce a new undergraduate teaching programme — technically under the school of humanities — in areas ranging from the history of science and technology to “science, technology and the organisation of industrial society”. A fellowship programme begins next year.

From the beginning, those responsible for the programme have emphasised that a high academic standard is necessary to avoid the ‘credibility’ problems associated with many previous attempts at multidisciplinary education. At the same time, however, it is stressed that the programme is also to be of direct use to the future scientist or engineer.

The 1977 prospectus for the college, for example, emphasises that the students will acquire “marketable knowledge”, and that they will be “more effective professionals for this knowledge”.

“The trends in terms of public perception of the implications of science and technology for society are continuing to mount, even if students are less vocal about it than they were at the beginning of the decade,” says Dr Donald Blackmer, director of the new programme and deputy dean of the school of humanities. “The converse of this is that scientists and engineers are increasingly concerned about the impact of government and public attitudes on what they do.”

This impact is felt both on the application of science, and on its practice. Dr Wiesner, for example, has been an outspoken critic of the extent to which social controls manifest as government regulation may be holding back both industrial innovation and university research. Addressing a meeting of the National Council of University Research Administrators in Washington earlier this year, he stated that “In the technical realm we can see many ways to continue humanity's forward march, if we do not





*Behind MIT's new image: President Jerome Weisner (right) and his successor-to-be, Dr Paul Gray (centre). Left: chairman of the corporation of MIT, Howard W. Johnson*

put too many shackles on those elements of society — universities, business, even some parts of government — that have made our technological achievements possible.”

Closer to home, MIT biologists have had direct experience of the fierce and acrimonious debates that have surrounded the use of recombinant DNA techniques. Many were involved in public discussions over the extent to which the City of Cambridge should be permitted to exercise controls over such research.

Some now argue that it was a mistake ever to have raised publicly the potential hazards of this work. But Professor Gerald Holton of Harvard University, a visiting professor for the STS programme, argues that despite the “great cost and anguish”, the debate was a “historic necessity”, and that the main dangers lie not in attempts to restrict research as such, but the fact that they may be imposed from outside through an “uninformed bureaucracy”.

“Scientists should not be pushed into a defensive position, when it has been the scientists who have historically dealt with self-imposed limitations, such as the discipline of the peer-review process. They should be aware that including these new issues is part of an on-going maturing of the profession” Professor Holton says.

Thus a general concern, not so much the need to control the uses of science as the institutional forms through which society has chosen and is likely to choose to exercise this control, forms a backdrop to the activities of MIT's new programme. In terms of study groups and lecture series, these range from topics such as technology and work, or risk and regulation, to environmental studies and the role of computers in society.

This concern is also directly linked to an issue which has caused considerable discussion within the programme faculty, namely the extent to which it should get explicitly involved in issues of public policy. Initially there was a certain reluctance to do so from anything more

than a long-term, “basic research” perspective, a tendency illustrated by the high proportion of historians among initial faculty appointments.

More recently this emphasis has shifted, largely under promoting from Dr Wiesner, who stresses the “unique contribution” that universities can make to problems “that require mixed solutions that are at once technical, managerial, political and social.” Dr Blackmer talks about bringing in more policy people as helping promote “a useful dialogue”; Professor Walter Rosenblith, MIT provost and, like Dr Wiesner, a fervent supporter of the college, speaks of the advantages of maintaining a “creative tension”. But moves towards the policy direction — natural as they may be for an institution with such close links to Washington policy-makers as MIT — have also exacerbated tensions with radical critics who, while initially keen that MIT should expand its efforts in the “science and society” area, now express concern about the way that this is being done. “It is certainly very important to study these issues, but establishing a research programme is no substitute for responsible social action on the part of scientists and technologists; the message of the college is that these controversies are matters for academic experts,” says Dr Jonathan King, professor of biology and an active participant in recent DNA controversies.

Dr Blackmer expresses concern about the criticism that individuals who have been raising these issues on the campus have been excluded from the programme. But he defends this as the result of the need for a high level of academic credibility.

Here, too, however, there is a source of controversy. Dr Wiesner and Dr Rosenblith — both of whom have been asked to join the programme after their respective retirements next year — have publicly argued that universities can bring rationality to public debates about the risks of technological developments.

“University research, with its capacity to be comprehensive and with a credibility

based on objectivity, can play an especially important role in studies aimed at clarifying for government the elements of conflicting social goals,” says Dr Wiesner.

Critics, however, pointing to factors such as the sustained high profitability of chemical companies claiming to be overburdened by regulation, argue that the current ‘debates’ about risk are being partly managed as a public relations exercise by industry as part of a more general attack on social controls — and that MIT's academic credentials are being used to legitimate such tactics.

“There is a growing danger that, by recruiting university scientists to study these problems, private corporations are consciously trying to immunise themselves from public scrutiny by invoking both the scientists’ stance of neutrality and objectivity, as well as the more general academic respectability of interdisciplinary programmes such as STS”, says Dr David Noble, an associate professor of history.

Dr Stephan Chorover, professor of brain sciences and a long-term campus activist, puts it even more strongly: “The bottom line is that the college is an exercise in what I call the engineering of consent: the product of a marriage between corporations with a large amount of money and low public credibility, and an institution that lacks the money but can provide the credibility.”

Such criticism is not confined to plans for the college; another target is a new proposal, eagerly supported by some faculty members but criticised by others, that MIT should set up a “risk institute” largely at the behest of industry specifically concerned with technological risks and regulatory responses.

Given MIT's pre-eminence in what STS faculty member Dr Leo Marx has described as “the institutional embodiment of perhaps the major theme in the development of Western culture since, roughly, the seventeenth century”, the outcome of the various debates will be watched with particular interest. □

# news and views

## Pathways to secretion

by Peter Newmark

OF the complex series of events that culminate in the secretion of products from cells, most can still only be classified as phenomenology in the view of G. Palade (Yale University), expressed at a recent meeting.\* Although several attractive candidates are currently vying for recognition as molecular mechanisms of secretion, so far that accolade has only been conferred on the signal hypothesis.

The signal hypothesis implies that proteins destined for secretion are synthesised with a sequence that guides them (and the ribosomes on which they are being synthesised) to the membrane of the endoplasmic reticulum (ER). As synthesis proceeds the nascent protein passes through the membrane into the lumen of the ER and from there, eventually, to and/or through the external membranes of the cell. Much evidence favours this concept, especially in the form in which the signal is a short, predominantly hydrophobic, amino-terminal sequence which is cleaved off as the protein traverses the ER. V. Lingappa (Rockefeller University) made the point that the concept could survive examples of non-terminal signals thereby rationalising the evidence he presented for the existence of an internal and non-cleaved signal sequence in ovalbumin (see *Nature* 281, 117; 1979).

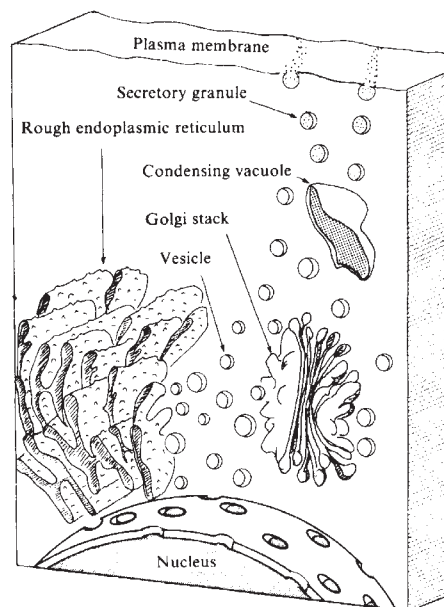
One prediction of the signal hypothesis is that the addition of a signal sequence to a non-secreted protein will lead it to be secreted. Another is that without the proper signal sequence a secretory protein will not be secreted. J. Beckwith (Harvard Medical School) described experiments with *Escherichia coli* mutants that have borne out both predictions. To test the first, genetic recombination was used to replace the amino-terminus of  $\beta$ -galactosidase (a cytoplasmic protein) with the amino-terminal sequences of either the maltose-binding protein (found in the periplasmic space between the inner and outer membranes of Gram negative bacteria) or the phage lambda receptor (an outer membrane protein). Either grafted signal sequence had the predicted effect of

redirecting  $\beta$ -galactosidase from the cytoplasm to within, if not through, the bacterial membranes.

The *E. coli* with mutant  $\beta$ -galactosidase stuck in their inner cell membrane became phenotypically abnormal when induced with maltose. This led to confirmation of the second prediction of the signal hypothesis. For it was possible to isolate pseudorevertant bacteria which did not assume an abnormal phenotype when induced with maltose. These pseudorevertants did not have membrane-bound  $\beta$ -galactosidase. Genetic mapping showed this to be due to a mutation in the signal sequence region. Genetic recombination enabled the mutated signal sequences to be fused back to their normal proteins, maltose-binding protein or the lambda receptor; whereupon they were no longer secreted. Finally the exact mutations of the signal sequences that prevented their functioning were determined at the DNA level and sometimes at the amino acid level too. In most cases the change was of a hydrophobic to a charged amino acid.

Once across the rough endoplasmic reticulum and into the cisternal space, proteins are *en route* to secretion. But not all of them become secreted. In particular, certain enzymes are destined instead for the lysosomes. W. Sly (Washington University School of Medicine, St Louis) suggested that lysosomal enzymes are diverted from secretion by means of another signal, a mannose-6-phosphate group on a complex oligosaccharide group that is added to the enzymes in the cisternal space. The mannose-6-phosphate then acts as a recognition marker for receptors on the cisternal side of the endoplasmic reticulum membranes, the ligand-receptor complexes being internalised into vesicles which become lysosomes. These events, Sly believes, take place before the proteins reach the Golgi complex. If the mannose-6-phosphate recognition marker is missing from lysosomal enzymes they are secreted instead; this being the molecular basis of I-cell disease.

Although an exposed mannose-6-phosphate group may be unique to lysosomal enzymes, the presence of an



Secretory pathways in the cell (adapted from *Functional Histology* (Wheeler, Burkitt & Daniels) page 12, Churchill Livingstone).

oligosaccharide group is a common feature of secreted proteins. The groups are synthesised on a lipid (dalachol) carrier, added to the proteins during or shortly after translation, and processed before or in the Golgi complex (M. Snider, Massachusetts Institute of Technology). Although the role of these oligosaccharides is not clear it is possibly in the routing of the proteins to their final destination. A genetic approach to this problem may be found through the availability of temperature-sensitive mutants of vesicular stomatitis virus, the spike glycoproteins of which do not reach the surface of infected cells at the non-permissive temperature. Some of the glycoproteins do not acquire normal terminal sugars on their oligosaccharide side chains, although at least one does (H. Lodish, Massachusetts Institute of Technology).

Molecular mechanisms which are tentatively related to secretion were outlined by H. Pollard (NIH) and T. Stossel (Massachusetts General Hospital). Pollard described a protein, synexin, which can aggregate isolated chromaffin granules

\*Basic Mechanisms of Cellular Secretion, sponsored by the NIH and held in Annapolis, 17-21 September, 1979. A book based on the conference will be published by Academic Press in 1980.

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in the presence of calcium. By analogy he believes that an influx of calcium into the cell would induce synexin-mediated fusion of chromaffin (or other) granules to the plasma membrane. For discharge of the contents of the granules another mechanism is needed. That, according to Pollard, involves the opening of an anion transport channel at the point of contact between the vesicle and plasma membrane leading to a chloride and energy-dependent chemiosmotic lysis of the granule. Stossel has a scheme whereby calcium activates the macrophage protein gelsolin (*Nature* **281**, 583; 1979) to reduce the length of the actin polymers of the cytoskeletal lattice. As a result he envisages that an influx of calcium could induce a local contraction of the lattice allowing access of the secretory granules to the plasma membrane.

Both of these speculative mechanisms involve triggering by an increase in the level of calcium ions, one of the best established features of stimulus-induced secretion. O.H. Petersen (Dundee University) described evidence from the pancreatic acinar cell that suggests the immediate release of calcium from an internal source in response to secretagogue stimulus. That calcium mediates the opening of ion channels for sodium and chloride, the influx of which accounts for the observed membrane depolarisation which is followed by secretion. Continued secretion requires the influx of calcium from an external source. That calcium may be brought into the cell by an ionophore. Candidate ionophores are phosphatidic acid (J. Putney, Wayne State University School of Medicine) and, at least in polymorphonuclear leukocytes, prostaglandin B<sub>x</sub> (G. Weissmann, New York University School of Medicine).

The question of the route(s) of intracellular transport of secretory products from the rough endoplasmic reticulum to the plasma membrane remains vexed. The favoured scheme is one whereby vesicles are pinched off from the former and find their way to the distended ends of the Golgi stack. With these they fuse. What happens next is unclear but may involve passage of the secretory products to other sections of the Golgi complex, the GERL (several participants felt that this was no longer a useful concept) and the condensing vacuoles. Finally, from one or more of these morphological semi-entities, vesicles are again pinched off. These become the secretory granules as they head for the plasma membrane. What, if anything, guides or drives them there? One possibility for which the evidence is ambiguous, is that microtubules are involved. If so, it is conceivable that secretory granules are attached to the tubulin subunits of microtubules which, according to L. Wilson (University of California, Santa Barbara), are continually being added at one end, traversing the length and disassembling at the other end of microtubules of constant

length. His latest evidence is that this 'treadmilling' is hastened by ATP, perhaps through the phosphorylation of one of the microtubule-associated proteins. The rate of treadmilling is thereby of the right order of magnitude were it to account for the conveyancing of granules to the surface of the cell.

Finally the secretory granule reaches the plasma membrane. Fission in the pancreatic B cell is preceded, according to L. Orci (University of Geneva) by the clearance of intramembranous particles and glycoproteins from the contiguous granule and plasma membranes. This is an observation common to many membrane

systems but was disputed by T. Reese (NIH) for *Limulus* amibocytes observed with a time resolution of a few milliseconds. Orci also has very tentative evidence that the intramembranous particle-free membrane areas are rich in cholesterol, on the fiercely disputed assumption that it is aggregates of cholesterol which form the protuberances that are induced by addition of the antibiotic, filipin. These morphological correlates of exocytosis seem to be mirrored by an absence of filipin-induced protuberances and a concentration of intramembranous particles at sites of endocytosis on fibroblast membranes □

## Jets in radio galaxies

from R.D. Davies

As higher angular resolutions become available to radio astronomers more evidence is coming to light on the important role of jets in the processes within radio galaxies and quasars. One of the first such objects to be discovered was the radio jet in the nearby radio galaxy Virgo A, also known as M87, the dominant galaxy of the Virgo cluster. This jet, seen at both optical and radio wavelengths, is a straight filament aligned precisely with the nucleus of M87; it lies to one side and extends 1.5 kiloparsecs (4,500 light years) from the nucleus. A similar geometrical arrangement occurs in the quasar 3C273, but on a larger scale; a straight jet projects 40 kiloparsecs from one side of the compact optical and radio quasar. The discovery of such jets lying relatively close to the nucleus of radio galaxies and quasars gave an impetus to the understanding of the huge outer radio emitting lobes. These are generally of similar intensity and lie a similar distance either side of a central object; the overall dimensions may be anywhere in the range of 100 to 3,000 kiloparsecs.

The arrangement of radio sources (it does not particularly matter whether they are radio galaxies or quasars) can be simply summarised. There are three main parts — a central power-house in which energy is released, an interconnecting region where energy is transmitted outwards and a large outer luminous region. Since these outer regions lose energy by radiation in a shorter time than it took them to expand out to their present position, the energy supply must be continually replenished. Therein lies the problem. The situation is analogous to the problem of supplying energy to a city from a distant coal-field. We might think of two ways of doing this. First, the energy could be converted directly into electricity then sent over transmission lines, or alter-

natively the coal could be transported by train and converted locally to the form of energy required. The same two alternatives have been proposed for radio sources. Rees has proposed that energy may be transmitted as intense low-frequency electromagnetic radiation which then penetrates to the outer lobes and causes them to radiate. Other theories have been conceived in terms of the mechanical transport of energy in which either cold gas or relativistic gas and magnetic fields are ejected at high speed from the nucleus and interact with the material in the outer lobes. The latter processes involving the mechanical transport of energy are more likely to produce observable radio jets.

New radio instruments with even higher angular resolution and sensitivity are coming into operation and are throwing light on the radio source problem. The pioneering instrument in this field is the Cambridge 5 km aperture-synthesis array; it has been for many years a major instrument in the study of structure of radio sources. It is now equipped with receivers at short wavelength to improve further its angular resolution. The Westerbork array in Holland is being increased in resolution by an extension of baseline to 3.2 km. The major development in this field in recent years is the Very Large Array (VLA) which is nearing completion in Socorro, New Mexico. The VLA will consist of 27 radio telescopes each 25 metres in diameter located on rail tracks in the form of a Y with each arm 18 km long. Higher resolution again will be achieved with the Multi-Telescope Radio-Linked Interferometer (MTRLI) at Jodrell Bank which will have telescopes distributed over baselines up to 133 km. The highest resolutions of all (milliarcseconds) are available with tape-recording interferometers on intercontinental baselines; these are particularly useful in probing the nuclear regions of radio sources.

The new observations of radio sources

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with the partially completed VLA were a highlight of the recent General Assembly of the International Astronomical Union. 3C449 described by Perley, Willis and Scott (see *Nature* **281**, 437; 1979) is typical of the new data coming from the VLA. This is the radio source associated with a giant elliptical galaxy having a redshift of 5,400 km s<sup>-1</sup>. The radio galaxy is typical of its class and has large outer radio lobes extending to 160 kiloparsecs on each side of the galaxy. What is particularly evident from the new results is the dominance of an inner jet structure lying symmetrically on either side of an unresolved nuclear source. These jets show a constant opening angle of about 7° which is most readily interpreted as arising from fast radial outward flow of emitting plasma in which there is also a transverse flow representing expansion at the speed of sound on the medium. A consistent picture emerges with a radial flow of about 1000 km s<sup>-1</sup> which leads to a lifetime of 10<sup>7</sup> years for the bright inner jets.

In addition to the bright jets and the faint but extensive outer lobes there is an intermediate region of enhanced brightness. In all, there must have been three major outburst phases in 3C449, in each of which material was ejected outwards in opposite directions on more or less the same line. As the emitting plasma moves outwards it expands and becomes of lower surface brightness. Indeed the authors interpret the gap between the nucleus and the inner edge of the jet as indicating that there may have been an interval since the last outburst of a few million years. These observations clearly favour an explanation in terms of a mechanical flow of energy in the form of a plasma of relativistic particles and magnetic fields. The details of the geometry, the polarisation and the spectral index of the emission along the jets will be important in the full understanding of the physical mechanisms in radio galaxies. □

## Bent DNA

from Michael Spencer

FOR many years after the initial excitement wore off, the devotees of DNA stereochemistry soldiered on with their studies amid some disbelief that the detailed results would be of much interest to those working on biochemical problems. It was an esoteric speciality, occupied with the niceties of sugar ring pucker, base tilt and base twist. All this began to change as biochemists accumulated evidence for non-coding regions of DNA containing special sequences, in particular the promoter region associated with the binding of RNA polymerase. It appeared

in this case that the binding caused a localised unwinding of the DNA duplex, and interest grew in discovering exactly what changes in structure were involved. In this way it was hoped to obtain a better understanding of how the enzyme recognised the site and initiated synthesis.

In two other fields DNA deformation became of interest, and models were proposed for a 'kink' involving the unstacking of one base pair relative to an adjacent pair. It seemed a plausible way of explaining the compaction of DNA in the nucleosome (Crick & Klug *Nature* **255**, 530; 1975), though other interpretations were later suggested (see *News and Views*, 1 September 1977). In the field of DNA-drug interactions, it was proposed that intercalation of a drug molecule could occur at sites where a spontaneous kink, induced by a thermal energy fluctuation, allowed the drug to slip in between the base pairs (Sobell *et al.* *Cold Spring Harbor Symp. quant. Biol.* **42**, 87; 1977). It was further suggested that such a kink might propagate down the polymer in a wave like disturbance.

In all this, it was generally assumed that DNA remained in the well-known *B* conformation that purified material shows in an aqueous environment. However, a few workers always hoped that they would some day find a biological role for the rather different *A* conformation that is often found under conditions of low water content and low ionic strength. This has 11 instead of 10 base pairs per turn, and the bases are tilted and laterally displaced in relation to the helix axis. It has been known since 1962 that RNA had a structure of the *A* type, and the *A* structure received a further boost when it was discovered that DNA-RNA hybrids preferred it. The idea germinated that even if DNA *in vivo* did not adopt an *A* structure along its whole length, there might be local transitions induced by changes of environment or interaction with other, more hydrophobic molecules (Arnott *et al.* *Nature* **220**, 561; 1968; Florentiev & Ivanov *Nature* **228**, 519; 1970). More recently, Minyat *et al.* (*J. molec. Biol.* **128**, 397; 1978) have obtained evidence from circular dichroism that spermine and spermidine do stabilise the *A* conformation in solution, under conditions of decreased water activity. They deduced that the cooperative unit could be as short as 10 base pairs.

A collaboration between groups at Wisconsin and Purdue (Selsing *et al.* *J. biol. Chem.* **254**, 5417; 1979) has now led to a convincingly detailed model for a DNA duplex containing an *A* structure at one end and a *B* structure at the other, with a sharp bend in the middle where the conformation changes. Their work follows the synthesis of a model copolymer containing dG along the whole of one strand; the other strand contains a block of rC, giving the *A* structure characteristic of hybrids, linked by T4 DNA ligase to a section of dC, which in solution leads to a *B*

structure. Solution studies by Selsing and Wells (*J. biol. Chem.* **254**, 5410; 1979) have confirmed the earlier conclusion from NMR work that the transition region is confined to only one or two base pairs.

The new model-building exercise was carried out for a purely DNA duplex with only a single nucleotide pair in the transition region. In the most satisfactory of the models tested, the transition base pair has one nucleotide with the sugar pucker characteristic of *A*-DNA, and one with the different pucker found in *B*-DNA. The change of conformation leads to a 26° change in the direction of the helix axis, while preserving full base stacking and hydrogen bonding. It may thus be energetically preferable to an unstacked kink. Furthermore, the base stacks predicted at the junction for different DNA sequences have noticeable similarities to those actually found in dinucleotide and polynucleotide structures.

The authors discuss the biological implications with some enthusiasm. Their model gives respectability to the belief that segments of *A*-DNA may be induced to form in an otherwise *B*-DNA helix, and this might well explain the observed unwinding of DNA on binding RNA polymerase. However, they do not go into the question of how the RNA might then be copied; does the helix open up, as originally suggested by Florentiev and Ivanov, or is a triplet structure formed as suggested by Riley (*Nature* **228**, 522; 1970)? Another relevant matter which they do mention is the discovery of alternating A, T-rich and G, C-rich blocks in many regulatory regions. In DNA fibres, the former type of sequence leads to a preference for a *B* conformation, while the latter extends the stability range of the *A*. If these preferences held under cellular conditions (which remains to be proved), the DNA could adopt a tertiary structure determined by the length of the segments. Alternating single turns of each kind of helix, both containing about 10 base pairs, would result in the helix axis following a zigzag path. On the other hand, half a turn of each helix would result in a small loop of DNA, and the authors illustrate a computer-generated model for such a structure.

If such features were stable *in vivo* they might form recognition sites for protein ligands; alternatively, they might be induced to form by the more hydrophobic environment resulting from the presence of protein. It is an intriguing thought that an enzyme working its way down the DNA duplex might trigger off a local change in conformation that stopped it in its tracks. The hunt must now be on for direct evidence of such local changes in structure. The authors point out that a loop containing *A*-DNA segments will contain a distinctly shorter average length per nucleotide than the superficially similar structure obtained by smoothly bending *B*-DNA. This could, they say, explain a

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recent observation by Griffiths (*Science* 201, 525; 1978) that electron micrographs of  $\phi$ X174 DNA suggest a nucleotide repeat of only 2.9 Å, instead of the 3.4 Å expected for B-DNA. The alternative explanation preferred by some workers is the existence in solution of a B-like structure with slightly more than 10 residues per turn (Wang *Proc. natn. Acad. Sci. U.S.A.* 76, 200; 1979).

The authors finish with a flourish of speculation about the possibility of travelling disturbances. The idea that DNA can 'breathe' and develop a travelling region of denaturation is an old one, and the idea of travelling kinks has already been mentioned; it now seems possible that travelling discontinuities between regions of different DNA conformation might be important in biological recognition. The sequence-specific conformational preferences that have been shown for DNA would, they say, act as 'sources, sinks and barriers' for transient conformational discontinuities in the helix. The idea of DNA as an inert repository of genetic information seems really to have breathed its last. □

## Locust phase colouration

from Richard J. Moore

CHANGE in colouration is the most noticeable feature of the 'phase' transformation which locusts undergo during their large population fluctuations, yet its adaptive significance has proved one of the most difficult to explain. Individuals in low population densities during plague recessions (*solitaria* phase) are a uniform green, presumably a disguise from the attentions of visually-guided predators. During plague outbreaks, individuals develop a striking black and yellow or orange colouration (*gregaria* phase).

This suggests an aposematic or warning function to predators; yet locusts are voraciously pursued by flocks of birds, and nomads regard them as something of a delicacy. Batesian mimicry of a poisonous or distasteful model is unlikely to be operating since no possible models with a similar range are known, and in any case the vast numbers of locusts in swarms would greatly outnumber any model.

Early research on the problem focused on the possible role of the *gregaria* colour pattern in maintaining migratory groups. In 1928, Boris Uvarov suggested that the increased activity of hoppers (nymphs) in bands resulted from an optomotor or visual compensation response to

movement of neighbours. Peggy Ellis (*J. exp. Biol.* 30, 214; 1953) found, however, that a background of moving vertical stripes was not as effective in maintaining the characteristic 'marching' of hoppers as visual stimuli from moving locusts. This and a more recent experiment by Sylvia Gillett suggests that locust shape is the more important stimulus for grouping. Gillett (*Anim. Behav.* 21, 153; 1973) mixed normally-pigmented *gregaria* desert locusts with albinos, which aggregate less than coloured individuals. Mixing them neither reduced the grouping of normal locusts, nor increased the grouping of albinos: thus the reduced grouping of albinos alone must be due to some other factor besides lack of colouring.

It should be noted that Gillett's experiment examined static aggregation; and also that the vertical stripes in Ellis's experiment were not specifically designed to imitate *gregaria* locust patterning. So it remains a possibility that the patterning has evolved to maximise the optomotor response, and thus cohesion of moving groups.

Recently, however, workers have begun to examine the role of predation as the evolutionary force promoting phase polymorphism; indeed, aggregation itself may give a defensive advantage against predators. Matthews proposes (*Amer. Nat.* 111, 213; 1977) that individual locusts, and also Australian stick insects (which undergo remarkably similar density-dependent phase transformations), contain small amounts of toxins, so that the consumption of large numbers of them by a predator would eventually exceed its tolerance limit. Remembrance of the strikingly-coloured prey which had produced the intoxication would result in a specific avoidance image (a negative 'search image') preventing further consumption of locusts. It is doubtful, though, how much protection would be conferred in this way, since large birds such as storks are known to be capable of consuming in the order of 1000 adult locusts per day, day after day.

Gillett has begun to look at the factors affecting locusts' vulnerability to predation. Against a green irregular background, surprisingly, *gregaria* nymphs are just as effectively camouflaged as *solitaria* (Gillett & Gonta *Anim. Behav.* 26, 282; 1978). But in a strategy of crypsis, density is also of major importance, as her experiments showed. In the wild, *gregaria* nymphs often occur massed in numbers so great as to preempt any possibility of escaping detection. These very numbers, and their increased activity, might make it difficult for a predator to pick off individual locusts. Could the *gregaria* pattern contribute to this?

In her latest work (Gillett, Hogarth & Noble, *Anim. Behav.* 27, 592; 1979), Gillett and her coworkers offered *gregaria* desert locusts at a range of densities to a lizard and human predators in an otherwise empty

arena. The catching efficiency of the human predators did not vary much, but the effect of increasing prey density on the lizard's catching efficiency was dramatic: above a certain prey density, the lizard became totally incapable of catching any locusts. Gillett *et al.* attribute these results to a 'visual confusion effect' produced by the activity of the locusts — an effect which, they suggest, would be enhanced by the bold patterning. This remains to be demonstrated, but there are strong indications of the importance of prey-stripping in other predator — prey interaction (see, for instance, the discussion in Milinski, *Z. Tierpsychol.*, 45, 373; 1977).

Although this is an attractive hypothesis, it may still not be the whole story. There is no reason why the *gregaria* colour pattern should not have multiple functions, but it is remarkable that neither of the most likely functions in locusts — group cohesion and predator confusion — could be of any use to Australian stick insects: they do not aggregate, and even at high densities, are very inactive. □

## New human myeloid leukaemia cell line undergoes red shift

from P. R. Harrison

BIOLOGISTS interested in elucidating how cells acquire their specialised functions (the process of cell differentiation) are always on the look out for ways in which these normal developmental processes can be mimicked by *in vitro* systems as these often allow the design of more sophisticated experimental approaches than are normally possible *in vivo*. This applies with even greater force of course to experiments with human cells. For a long time, cells from leukaemic patients or animals have provided useful models for how cells grow and differentiate and the relationship between the occurrence of malignancy and consequent abnormal cell growth and differentiation.

Recently a new human cell line has made something of an impact in the literature on account of its very interesting properties. The K562 cell line was established several years ago by Lozzio and Lozzio working at the University of Tennessee from the pleural effusion of a patient with chronic myeloid leukaemia (CML) in terminal blast crisis (*Blood* 45, 321; 1975). Initially it was assumed, largely on morphological criteria, that the K562 cells were derived from the outgrowth of a malignant clone of myeloid leukaemic cells arrested at some very early stage of granulocyte

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differentiation. By analogy with the now classic experiments in which mouse erythropoietic cell precursors infected with Friend virus can be induced to differentiate further by treatment with DMSO and other chemicals (Friend *et al. Proc. natn. Acad. Sci. U.S.A.* **68**, 378; 1971). Lozzio's group tested the effect of DMSO on the human K562 cell line but could find no evidence of enhanced differentiation (Bamberger *et al. Leukemia Res.* **2**, 305; 1978).

But this year the situation has changed dramatically with a series of recent reports. First, Anderson and his colleagues at the University of Helsinki found to their surprise that in fact the surface properties of the K562 cell line resembled red blood cells rather than granulocytes or their benign or leukaemic precursors and in particular K562 cells were found to synthesise a highly specific red blood cell membrane protein, glycophorin (*Int. J. Cancer* **23**, 143; 1979; *Nature*, **279**, 498; 1979). Further support for the idea that K562 cells are in fact erythroid precursor cells comes from the subsequent finding by Anderson and his colleagues that sodium butyrate can induce erythroid maturation in K562 cells as judged by the formation of erythrocyte-like particles and haemoglobin, assayed either by benzidine staining or immunologically (*Nature* **278**, 364; 1979). This conclusion has recently been taken one important and very interesting step forward by Rutherford and his colleagues in Oxford who have reported that haemin induces human embryonic haemoglobin in K562 cells, as judged by starch gel electrophoresis and comparison of fingerprints of tryptic peptides with those from authentic human embryonic haemoglobins (*Nature* **280**, 164; 1979).

Whether other inducers (for example, butyrate in the experiments of Anderson *et al.*) induce embryonic or adult haemoglobin in K562 cells is not yet clear. There is some precedent for this in the Friend cell where haemin induces preferentially a minor  $\beta$ -globin chain, rather than the usual major  $\beta$ -globin chain (Rovera *et al. FEBS Lett.* **81**, 366; 1977; Nudel *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 1100; 1977). Certainly, if different inducers were to induce different globin chains, then the K562 cell would provide an extremely interesting system for studying the embryonic to adult globin gene switch *in vitro*.

Nevertheless, the originators of the K562 cell line are evidently still not at all happy with this accumulated evidence for its erythroid nature since they cannot reproduce the induction of haemoglobin in their own original stocks of K562 cells by sodium butyrate which in their hands is merely cytotoxic (Lozzio *et al. Nature*, **281**, 709; 1979). Perhaps this is not too surprising since Rutherford *et al. (op. cit.)* found haemin to be the only effective inducer of K562 cells, butyric acid and other Friend cell inducers tested being ineffective at the concentrations normally

used. But those familiar with the Friend cell field will not be too perturbed at these differences between the laboratories concerning which inducers are most effective, since precise inducer concentrations and other variables in the culture conditions are often important. For example the batches of serum used, particularly in experiments where butyric acid is used as inducer, can affect the outcome. In any event, Lozzio *et al.*'s suggestion that the cells used by Anderson *et al.* (and presumably also Rutherford *et al.*) may have been contaminated with other cells with erythroid properties (a claim hotly and justly denied by Anderson *et al.* on the basis of karyotype evidence — *Nature*, **281**, 710; 1979) seems to me to be an inadequate explanation. Whether Anderson's karyotypically-authentic K562 line might have acquired its erythroid properties during clonal evolution in culture rather than *in vivo* is more difficult to resolve.

What then is the significance of the K562 cell line for the future? Is it a matter of *déjà*

*vu* and essentially a repeat of the Friend cell story in a human context? Not simply. The isolation of human leukaemic cell lines other than those derived from lymphoid precursors is relatively rare and therefore interesting especially when an adult cell can be induced to produce embryonic-type proteins. Since embryonic haemoglobins have not been found in association with CML the controversial origin of the cell is clearly of some considerable interest *per se*. Further, the existence of cell lines producing embryonic and adult globins (the K562 and Friend cells respectively) may allow the differential regulation of the embryonic and adult globins to be studied more thoroughly, for example, possibly by exploiting the effects of different chemical inducers or by cell fusion studies. And undoubtedly the K562 cell line will be exploited simply because it is a human erythroid cell line, for example it can be used for studies of the properties of human red blood cell precursors and as a source of embryonic globins and other red blood cell proteins. □

## Pituitary growth hormone

from Mike Wallis

THE pituitary growth hormones (GHs) together with the prolactins and placental lactogens comprise a family of protein hormones which regulate somatic growth, lactation and various other biological functions. An international symposium on GH and related hormones is held at four-yearly intervals in Milan; the most recent one\* was broadened to include 'Other Biologically Active Peptides' though fortunately this was not taken too literally, and GH and its relatives remained the main focus of attention (and will be the only topic considered here).

As in many other areas of biology, recombinant DNA technology is making a major impact on polypeptide hormone biochemistry. H.M. Goodman (University of California, San Francisco) described the cloning and characterisation of the gene for human GH. The gene includes at least two introns, and some evidence suggests that several slightly different (non-allelic) copies of the GH gene may exist in the human genome. The nucleotide sequence of the (cloned) cDNA has been determined, including the entire coding region for the preGH. Expression of immunologically active human preGH (as a fusion protein including part of the bacterial trpD gene product) has been achieved in *E. coli* and the production of

substantial quantities of human GH by genetic engineering in bacteria must now be imminent. The timing is appropriate since the hormone may well have new clinical uses (in addition to treatment of hypopituitary dwarfism); V. Zumtobel *et al.* (University of Munich) described applications of the hormone in the treatment of stress ulcers.

Another technique with great potential was described by J. Ivanyi (Wellcome Research Institute, Beckenham, UK) who reported the preparation and application of monoclonal antibodies to human GH. At least two antigenic sites can be recognised on human GH, only one of which is shared with human placental lactogen. This powerful technique should provide both specific probes for investigating structure-function relationships and reagents with increased specificity for radioimmunoassay.

Lack of detailed information about the three-dimensional structure of any member of the GH-prolactin family is a major drawback in understanding their biochemistry. The main problem is lack of suitable crystals for crystallography, and the reasons for this were discussed by K. Moffat (Cornell University). Crystals of human placental lactogen have been obtained but were insufficiently ordered for X-ray analysis. Heterogeneity of the hormone preparations that have been used for crystallisation attempts is a major problem, and Moffat urged those present to try and purify further, and to crystallise,

\*An international symposium on 'Growth Hormone and other Biologically Active Peptides' was held in Milan on 17-19 September. The meeting was organised by A. Pecile, E.E. Muller and M.L. Pecile and the Chairman was C.H.Li. Abstracts have been published (*Ricerca Scientifica ed Educazione Permanente Supplement 11*) and invited papers will be published in full by *Excerpta Medica*.



their favourite hormones.

In the absence of information about three-dimensional structure, knowledge about structure-function relationships must come from other sources. Species specificity is marked among the GHs (thus, non-primate growth hormones are not active in man) and comparisons of GHs from different species may eventually provide clues to structure-function relationships. Purification and characterisation of GHs from various lower vertebrates was described by H. Papkoff *et al.* (University of California, San Francisco). Immunological and physicochemical differences between the hormones mainly reflect the extent of phylogenetic divergence, but biological activities are rather variable and do not follow phylogenetic relationships so closely. Sequence information for these proteins is eagerly awaited, but for the present our knowledge of the molecular evolution of this protein family remains based largely on the sequences of mammalian hormones. Studies on chemically modified forms of GH, and on synthetic fragments, may also help relate structure to function, and were described by A.C. Paladini *et al.* (University of Buenos Aires) who are attempting to define precisely which residues are required for activity. At least one antigenic site has been tentatively identified as associated with a region of 30–50 amino acid residues long, near the centre of the primary structure.

The mechanism of action of GH remains unclear. In particular it remains uncertain (and a matter of considerable controversy) as to which of the actions of the hormone are direct and which are mediated by somatomedins. Like other polypeptide hormones, GH is thought to bind initially to a membrane receptor, and the nature and characterisation of receptors was discussed in several papers. Direct actions of the hormone on erythroid colony formation *in vitro* were described by D.W. Golde (University of California, Los Angeles) and direct actions on amino acid uptake and incorporation by diaphragm *in vitro* were discussed by K. Albertsson-Wikland, A. Lindahl and their colleagues (University of Göteborg).

However, most of the discussion about the actions of GH centred on the somatomedins and their role in mediating these actions. Various forms of somatomedin have been described, at least some of which are very similar to the insulin-like growth factors IGF 1 and IGF 2, which have now been quite well characterised and which were discussed by J. Zapf and E.R. Froesch (University Hospital, Zurich). J.J. Van Wyk *et al.* (University of North Carolina) described studies on the characterisation of human somatomedin C, including determination of a partial amino acid sequence; the hormone is similar to IGF 1. Somatomedin C stimulates growth of various types of cell *in vitro* and was also shown to have *in vivo*

## The physics of biology

from H. Eisenberg

THE business-like mood at EMBL's Hamburg laboratory at the DESY synchrotron installation was a welcome change from the *fin-de-siècle* recreational workshop atmosphere one sometimes encounters at fashionable watering places. At the recent EMBO-EMBL workshop\* held there, hope, progress, expectation, and fulfilment in the biological applications of neutron and X-ray scattering, in particular with synchrotron radiation, were discussed in depth.

The ILL neutron reactor in Grenoble has now been active for several years and an EMBL outstation, to provide the infrastructure for experiments in biology, was established some years ago. Synchrotron radiation provides information additional and complementary to the neutron scattering and, in particular, the high intensity and pulsed nature of the beam presents unique opportunities. The EMBL laboratory in Hamburg, directed by Heinrich Stuhmann, has made great strides in the continuing development of suitable facilities to explore these opportunities. Also, construction of new facilities for chemistry and biology at the DORIS storage ring in Hamburg is now underway and, in large measure, success will depend on the concomitant development of appropriate two-dimensional fast detector systems.

Sessions at the workshop reflected the varied interests of the workers involved. Muscle and collagen are two systems long studied by both X rays and neutron fibre diffraction. In particular, hope was expressed that considerable reduction of

data collection time into the millisecond range, in the case of synchrotron radiation, will enable performance of dynamic studies and characterisation of individual states in the contractile systems. In solution studies a major tool is the application of contrast variation, particularly favoured by the dramatic difference in neutron scattering between hydrogen and deuterium (with little change in structural or functional properties); complementary information is provided in the case of X-ray scattering by the use of the more classical contrast variation media such as sucrose or salts. These and other theoretical aspects were discussed, as well as the possibility of obtaining information-rich three-dimensional reconstructions from the analysis of spatial fluctuations from random systems. This should be useful for complex biological systems which have not been obtained in crystalline organised form, useful for X-ray diffraction. Problems related to anomalous scattering were also discussed.

Protein-nucleic acid interactions were discussed in sessions devoted to progress in the study of ribosomes, chromatin and viruses. In chromatin we are gaining more understanding on the higher order structures of solutions of finite chains of nucleosomes in a variety of experimental conditions. Additional major topics of discussion involved membranes and lipoprotein complexes, as well as proteins and protein assembly complexes, such as microtubule structure.

The close encounter between about eighty molecular biologists, theoreticians and instrumentalists led to a frank exchange of ideas, created new and extended existing paths of communication in a field of great topical interest.

\*The second EMBO-EMBL workshop on 'X-Ray and Neutron Scattering of Biological Structures' took place in Hamburg on 24–28 September, 1979.

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actions on the frog lens epithelium. Studies on somatomedin A were described by A. Skottner *et al.* (A.B. Kabi, Stockholm) though amino acid sequence data are still not available for this hormone, and its relationship to the IGFs is uncertain. Somatomedin B, is now thought to be without somatomedin-like properties, and therefore inappropriately named. Although somatomedins A and C are thought to mediate at least some of the actions of GH, and although they have many biological effects *in vitro*, attempts to show GH-like actions of somatomedins *in vivo* have previously met with little success. However, J.V.L. van den Brande, S.C. van Buul-Offers and their colleagues (Wilhelmina Children's Hospital, Utrecht) reported that clear-cut growth hormone-like effects (including increase in body weight and length) have now been obtained

when human somatomedins were injected into hypopituitary dwarf mice. Somatomedins are thought to be produced in the liver in response to stimulation by GH and other factors. Studies on the biosynthesis of somatomedin in cultured Buffalo Rat Liver cells were described by S.D. Schalch *et al.* (University of Colorado) who presented evidence for a precursor of the hormone of molecular weight about 32,000. Other hepatocyte systems which produce somatomedins (and also their binding proteins) were also described (G. Haselbacher *et al.*, University of Zurich; M. Binoux *et al.*, Trousseau Hospital, Paris); stimulation of somatomedin synthesis by GH was claimed. □

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# articles

## Distortions of the cosmic microwave background spectrum by dust

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*The cosmic microwave background spectrum supports the idea of a pre-galactic generation of stars. The distortions of the background spectrum from a black body can be due to emission by dust if a modest fraction of the mean cosmological density is present as silicate particles at epochs corresponding to redshift  $z \geq 100$ . Whether the entire background can be explained as emission from dust is investigated.*

In the standard hot big bang cosmology, the Jeans mass at the epoch of decoupling of matter and radiation ( $z \sim 1,000$ ) is about  $10^6 M_\odot$ , so that any strong density perturbations present on this scale would collapse rapidly, in  $\sim 10^6[(1+z)/1,000]^{3/2}$  yr. Dicke and Peebles<sup>1</sup> proposed that globular clusters might have formed in this way before galaxies did. White and Rees<sup>2</sup> suggested a more radical version of this idea, in which an early generation of stars, perhaps formed by these  $10^6 M_\odot$  objects, evolved rapidly to form dark remnants (presumably black holes or stars of very low mass) and now comprise the 'missing mass' postulated to bind clusters of galaxies and to make up haloes around massive spiral galaxies<sup>3</sup>. The concept of a pre-galactic generation of stars, often referred to as 'Population III', was taken further by Rees<sup>4</sup>, who suggested that the cosmic microwave background radiation could be the light from such stars absorbed and re-thermalised by dust grains, molecules or ionised gas. Various earlier schemes<sup>5-13</sup> for a non-cosmological origin of the cosmic background radiation have encountered difficulties, because of the severe requirements of energetics and thermalisation. The hypothesis of Rees minimises these problems, and may lead to an explanation of the observed average number of photons per baryon in the Universe, an unexplained constant ( $\sim 10^9$ ) in the standard big bang models.

### The effect of dust in the early Universe

We investigate here the effect of dust in the early Universe on the cosmic microwave background, including the effect of a pre-galactic Population III generation of stars. We assume that at epochs such that  $z \gg 100$ , the cosmic background has a black-body spectrum of temperature  $T_r = T_0(1+z)$ . In Rees' model<sup>4</sup>,  $T_0 \ll 3$  K, but in more conventional models  $T_0 \sim 2.7-3.0$  K. Suppose that at an early epoch a generation of stars is born that live for a time  $t_s$ , dying at epoch  $t_t$ , where  $t_s \ll t_t$ .

Let  $\epsilon$  be the efficiency with which the matter in the Universe is converted into radiation by this Population III. If most of the matter in the Universe forms into Population III objects and

undergoes conversion to helium and heavy elements, then  $\epsilon$  can be as high as  $\sim 0.01$ . If most of the energy is generated by accretion of material onto black holes, then  $\epsilon$  could be as large as 0.1–0.3. Conventional models for quasars evidently require such a high efficiency<sup>14</sup>.

Assuming the Population III objects radiate as black bodies with temperature  $T_s$ , radius  $r_s$ , and have number density  $\eta_s$  at epoch  $t_s$ , then

$$\epsilon \rho_m(t_s) c^2 = 4\pi r_s^2 \eta_s \sigma T_s^4 t_s \quad (1)$$

where  $\rho_m(t)$  is the matter-density at epoch  $t$ .

The energy-density in radiation is enhanced by this radiation input by a factor

$$\beta = \frac{\epsilon \rho_m(t_s) c^2}{a T_0^4 (1+z_t)^4} = \frac{\epsilon \rho_m(t_s) c^2}{a T_0^4 (1+z_t)} = 420 \epsilon \Omega h^2 [100/(1+z_t)] (2.7/T_0)^4 \quad (2)$$

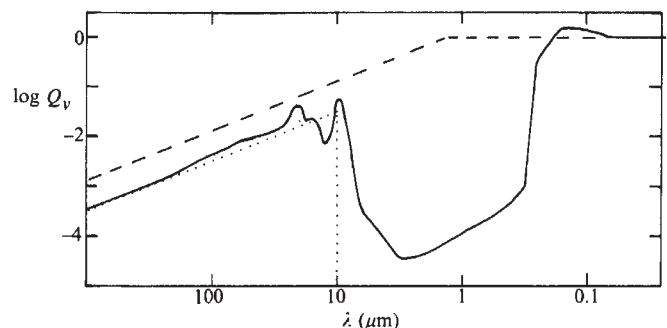
where  $\Omega$  is the cosmological density parameter, and  $h$  is the Hubble constant (units of  $100 \text{ km s}^{-1} \text{ kpc}^{-1}$ ).

Suppose that these stars give rise at their death to a heavy element abundance  $Z$  throughout the remaining gas in the form of dust. Presumably  $Z < 10^{-3}$  for consistency with the lowest values found in extreme Population II (ref. 15). If the dust is uniformly distributed, the resulting optical depth at epoch  $t_1$  to a source emitting at epoch  $t_2$ ,

$$\tau_\nu(t_2, t_1) = \pi a^2 c \int_{t_2}^{t_1} n_g(s) Q_\nu(s) ds \quad (3)$$

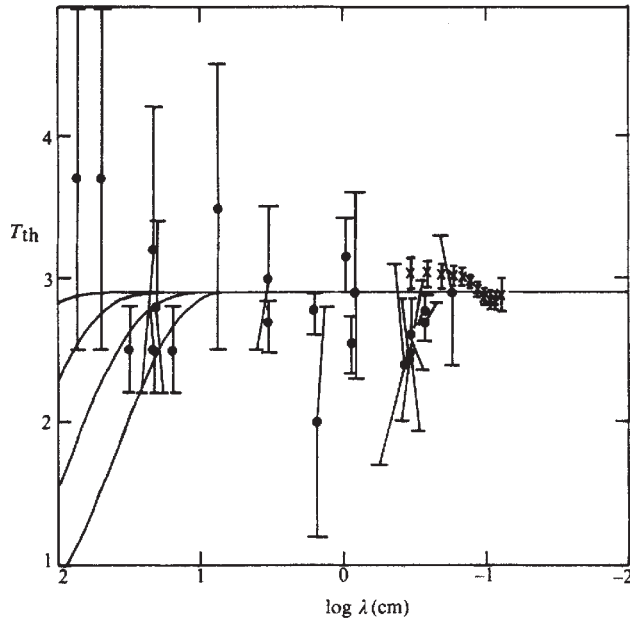
where  $n_g(t)$  is the number-density of grains at epoch  $t$ , and  $1+z = R(t_1)/R(s)$ . Thus

$$\tau_\nu(t_2, t_1) = A \int_{t_2}^{t_1} Q_\nu(s) [R_0/R(s)]^3 ds / t_H$$



**Fig. 1** Dust absorption efficiency used in this work. Solid line, amorphous silicates; dotted line, analytic approximation to these (equation (15)); dashed line, equation (14).





**Fig. 2** Observed microwave background spectrum (●, from Danese and de Zotti<sup>13</sup>; ×, from Woody and Richards<sup>20</sup>), plotted as thermodynamic temperature,  $T_{th}$ , against wavelength, compared with predictions for grains with  $Q_v$  given by equation (14),  $T_d = 2.9$ ,  $T_0 = 0$ ,  $1 + z_t = 200$  and (from the top)  $qZ = 10^{-2}$ ,  $4 \times 10^{-3}$ ,  $2 \times 10^{-3}$ ,  $10^{-3}$ .

where  $A = \pi a^2 n_g(t_0) c t_H$ ,  $Q_v$  is the absorption efficiency of the grains (as the radiation field in this problem is always isotropic, scattering can be entirely neglected),  $R_0 = R(t_0)$ , and  $t_H$  is the Hubble time at the present epoch  $t_0$ .

In terms of  $\Omega$ ,

$$A = 5.1 \Omega h (Z/0.001) (0.1 \mu\text{m}/a) (2.5 \text{ g cm}^{-3}/\bar{\rho}_g) \quad (4)$$

where  $a$  is the grain radius, and  $\bar{\rho}_g$  is the mean density of the grain material.

If  $T_d(t)$  is the temperature of the dust grains at epoch  $t$ , the intensity of radiation observed at epoch  $t > t_i$  is the sum of the contributions from the cosmic background and Population III, attenuated by dust, and from the emission by dust:

$$I_\nu(t) = \{(1 + z'_t)^{-3} B_{\nu(1+z'_t)}[T_r(t)] + (1 + z'_t)^{-3} \pi \eta_s r_s^2 c t_s B_{\nu(1+z'_t)}(T_s) \exp(-\tau_\nu(t, t)) + \int_{t_i}^t (1 + z')^{-3} B_{\nu(1+z')}[T_d(s)] \exp(-\tau_\nu(s, t)) d\tau_\nu(s, t)\} \text{ erg cm}^{-2} \text{ s}^{-1} \text{ H}^{-1}$$

where  $1 + z'_t = R(t)/R(t_i)$ ,  $1 + z'_i = R(t)/R(t_i)$ ,  $1 + z' = R(t)/R(s)$ . This can be written:

$$I_\nu(t) = \{B[T_0(1 + z)] + \pi \eta_s r_s^2 c t_s B_s[T_s/(1 + z'_t)]\} \times \exp(-\tau_\nu(t, t)) + \int_{t_i}^t B_\nu[T_d(s)/(1 + z')] \exp(-\tau_\nu(s, t)) d\tau_\nu(s, t) \quad (5)$$

where  $1 + z = R_0/R(t)$ .

The equation of radiative balance for the grains takes the form

$$\int_0^\infty Q_v B_\nu[T_d(t)] d\omega = \int_0^\infty Q_v I_\nu(t) d\nu \quad (6)$$

In particular, at  $t_i$  equation (6) becomes

$$\int_0^\infty Q_v B_\nu[T_d(t_i)] d\nu = \int_0^\infty Q_v B_\nu[T_0(1 + z_t)] d\nu + \pi \eta_s r_s^2 c t_s \int_0^\infty Q_v B_\nu(T_s) d\nu \quad (7)$$

As  $T_d(t_i) \leq T_m$ , the melting temperature of the grains, equation (7) implies

$$\pi \eta_s r_s^2 c t_s \int_0^\infty Q_v B_\nu(T_s) d\nu < \int_0^\infty Q_v B_\nu(T_m) d\nu \quad (8)$$

Assuming the stars radiate in the UV (for example, 0 stars with  $T_s \sim 40,000$  K) so  $Q_v$  can be set equal to unity on the left hand side, equation (8) is equivalent to a limit on the product  $\beta(1 + z)^4$ . If we set  $Q_v = 2x$ ,  $x = 2\pi a/\lambda$ , we find  $\beta(1 + z)^4 < 10^{10.65}(T_m/1,500)^5$ . If  $\beta = 1$ , as in Rees' model<sup>4</sup>,  $(1 + z) < 400$ . Before this epoch grains could not survive. For silicate grains, the limit is more severe,  $1 + z < 10^{2.03} \beta^{-1/4}$  for  $T_m = 1,500$  K.

### High optical depth limit

Suppose that at the present epoch, the optical depth in dust is large at all wavelengths  $\leq 20$  cm, say. Then the contribution of the first two terms on the right hand side of equation (5) to the integral on the right hand side of equation (6) is negligible, and the solution of equation (6) is

$$T_d(t) = T_d(t_0)(1 + z), \quad t \gg t_i \quad (9)$$

The observed intensity at the present epoch in the microwave range would be

$$I_\nu(t_0) = B_\nu(T_0) \exp(-\tau_\nu(t_i, t_0)) + B_\nu[T_d(t_0)] \times [1 - \exp(-\tau_\nu(t_i, t_0))] \quad (10)$$

To determine  $T_d(t_0)$ , we note that the conservation of co-moving energy density implies that

$$T_d(t_0)^4 \approx T_0^4(1 + \beta) \quad (11)$$

In particular if there were no radiation from stars ( $\beta = 0$ ), then  $T_d(t_0) = T_0$ , and  $I_\nu = B_\nu(T_0)$ , so that there is no distortion from a black-body spectrum. Equation (9) may not be a bad assumption where the optical depth in the UV is high, but that in the microwave region is  $\approx 1$ . This is because although  $T_d(t) \gg T_0(1 + z_t)$  in general, the temperature subsequently drops rapidly with time as  $\tau_{UV}(t, t)$  increases, until it is of the form of equation (9). However, in this case equation (11) should be replaced by

$$T_d(t_0)^4 [1 - \exp(-\tau_{\nu(T_0)})] \approx T_0^4 [1 - \exp(-\tau_{\nu(T_0)}) + \beta] \quad (12)$$

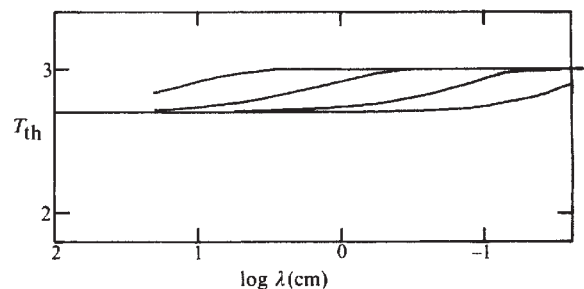
where  $\tau_{\nu(T)}$  is the optical depth back to  $t_i$  at the frequency corresponding to the peak of a Planck spectrum with temperature  $T$ . Thus if  $T_d(t_0) = T_0(1 + \delta)$

$$1 + \delta \approx [1 + \beta/(1 - \exp(-\tau_{\nu(T_0)}))]^{1/4} \quad (13)$$

For  $1 + z_t = 200$ ,  $T_0 = 2.7$ ,  $\beta = 0.25$  (for  $\epsilon = 0.01$ , this means  $\Omega h^2 = 0.12$ ),  $\tau_{\nu(T_0)} \sim 1$ , then  $T_d(t_0) = 3.0$  K.

### Assumptions about grain properties

The absorption efficiency in the IR of interstellar grains in the solar neighbourhood is not well known, so the properties of the



**Fig. 3**  $T_{th}$  against  $\lambda$  for grains with  $Q_v$  given by equation (14),  $T_d = 3.0$ ,  $T_0 = 2.7$ ,  $1 + z_t = 100$ , and (from the top)  $qZ = 10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$ .

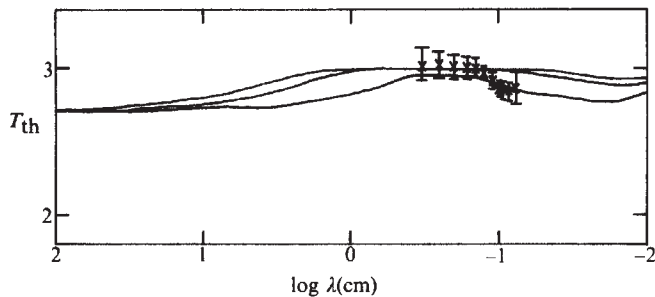


Fig. 4  $T_{th}$  against  $\lambda$  for amorphous silicate grains,  $T_d = 3.0$ ,  $T_0 = 2.7$ ,  $1 + z_t = 200$ , and (from the bottom)  $Z = 10^{-3}$ ,  $5 \times 10^{-5}$ ,  $10^{-4}$ , compared with Woody and Richards rates.

postulated grains in the early Universe can only be speculative. We have investigated three assumptions about  $Q_v$ :

$$(a) \quad Q_v = qx, \quad x = 2\pi a/\lambda < 1/q \\ = 1, \quad x \geq 1/q, \quad \text{where } q \leq 2 \quad (14)$$

With  $q = 2$  this represents the theoretical maximum efficiency for grains<sup>17</sup>. It has been argued<sup>16</sup> that in the far IR, grains in hot-centred molecular clouds have  $q \sim 0.1$  for  $a = 0.1 \mu\text{m}$ .

(b) Amorphous silicate grains, for which we have calculated the absorption efficiency in the range 9–200  $\mu\text{m}$  from the data of Day<sup>18</sup>, used the  $Q_v$  for terrestrial silicates given by Aannestad<sup>19</sup> for  $\lambda < 9 \mu\text{m}$ , and extrapolated assuming  $Q_v \propto \nu$  for  $\lambda > 200 \mu\text{m}$  (see Fig. 1).

(c) An analytical approximation to the  $Q_v$  for amorphous silicates, suitable for our purposes, given by

$$Q = 0.5 x, \quad \lambda \geq 10 \mu\text{m} \\ = 0, \quad 10 \mu\text{m} > \lambda > 2\pi a \\ = 1, \quad \lambda < 2\pi a \quad (15)$$

We have evaluated equation (10) for these types of grain with various assumptions about  $T_0$ ,  $T_d(t_0)$  and  $A$  (or  $Z$ ). We are interested in two cases in particular: first, Rees' model<sup>4</sup> to explain the whole microwave background radiation from grains; and second, the spectrum observed in the millimetre and sub-millimetre range by Woody and Richards<sup>20</sup>, which seems to show a significant distortion from a Planck spectrum.

We have plotted the results in the form of the thermodynamic temperature  $T_{th}$  as a function of  $\nu$ , defined by  $I_\nu = B_\nu(T_{th})$ . Figure 2 shows models with  $T_0 = 0$ ,  $T_d(t_0) = 2.9$ , for absorption efficiencies of the form of equation (14), with various values of  $qZ$ , and with  $1 + z_t = 200$ , compared with observations. As  $q \leq 2$  (ref. 17), and in practice probably  $q \leq 0.5$  (see ref. 19 and assumption (b) above), we see that the value of  $Z$  needed to fit the observations at long wavelengths is higher than that estimated by Rees<sup>4</sup>. We find  $Z \geq 0.001$ , and this exceeds the metal abundance seen in some globular clusters in our Galaxy<sup>15</sup>. This would imply that the matter which was to form into galaxies had to remain relatively unpolluted by the heavy elements made by Population III, an artificial situation. It is possible that some other material can be found to fill in the opacity at long wavelengths, for example, redshifted molecular lines. However, this model also fails to explain the distortion seen by Woody and Richards<sup>20</sup>.

Figure 3 shows models with  $T_0 = 2.7$ ,  $T_d(t_0) = 3.0$ , and grains of the form of equation (14) with various values of  $qZ$ , for  $(1 + z_t) = 100$ . Rather like the distortions caused by the effect of Compton scattering on heat input before decoupling<sup>21</sup>, we see a gradual changeover from  $T_{th} = 2.7$  at long wavelengths to  $T_{th} = 3.0$  at short wavelengths. This does not correspond with what is observed.

Figure 4 shows a range of models with amorphous silicate grains and  $T_0 = 2.7$ ,  $T_d(t_0) = 3.0$ ,  $1 + z_t = 200$ . The distortions from a black-body spectrum are rather similar to those observed

if  $Z \sim 10^{-5}$ . Figure 5 shows a range of models for grains with  $Q_v$  of the form of equation (15) and  $1 \geq z_t = 100, 200, 500$  (the latter case is implausible by equation (8)). The analytical approximation gives results very similar to those for amorphous silicates. The crucial requirement in explaining a distortion of the type seen by Woody and Richards<sup>20</sup> is the sudden drop in absorption efficiency at wavelengths  $< 10 \mu\text{m}$ . The redshifting of this drop in opacity into the millimetre range can give the observed distortions. None of the classical pre-decoupling distortions that have been predicted<sup>21</sup> can account for these distortions. No other grain material shows this phenomenon as strongly as silicates.

## Conclusions

The Rees model comes close to fitting the whole background spectrum but seems to be in trouble at wavelengths  $\geq 20 \text{ cm}$  if we are restrained to plausible values of the metal abundance at early epochs. It also fails to fit the Woody and Richards<sup>20</sup> data, as does the classical big bang picture with or without heat input before the decoupling era.

If the Woody and Richards spectrum is taken seriously, it can be explained as being due to emission by silicate dust at early epochs. The required metal abundance is rather low,  $\sim 10^{-5}$  by mass, and is consistent with limits obtained from the absence of reddening or a 2,200 Å absorption feature in the spectra of most quasars<sup>22</sup>. The energy input needed to distort the spectrum so drastically is substantial, requiring most of the matter in a Universe with the closure density ( $\Omega = 1$ ) to undergo thermonuclear reactions in Population III. There are essentially no free parameters in our model because although any  $z_t \geq 100$  would give the observed distortion, the grains would be melted at epochs such that  $z_t \geq 200$ . For the same reason the black-body spectrum that we assume to be present at epochs such that  $z \gg 100$  could not be the result of some earlier thermalisation by dust (say of radiation from black holes in the cold universe of Carr<sup>23</sup> or the tepid universe of Carr and Rees<sup>24</sup>).

We have not attempted to give any astrophysical details of the proposed Population III, as these details are not likely to be observable. One obvious requirement is that the dust be spread sufficiently homogeneously to be consistent with the small-scale isotropy of the background. As the dust has a strong effect only at  $\lambda \sim 1\text{--}3 \text{ mm}$ , this is not a very severe requirement at present. A search for small-scale structure in the millimetre background

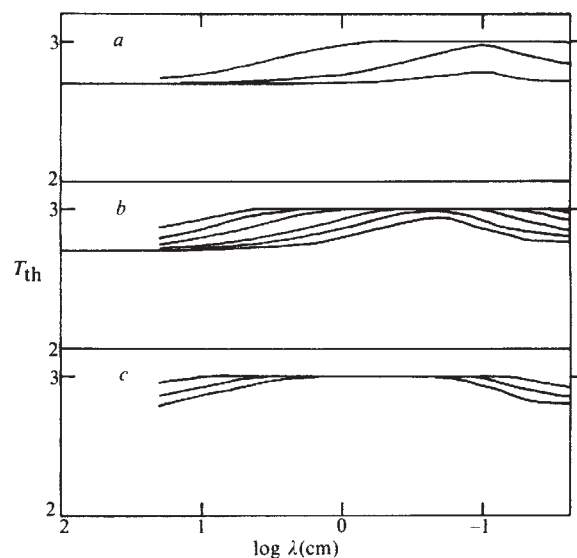


Fig. 5  $T_{th}$  against  $\lambda$  for grains with  $Q_v$  given by equation (15),  $T_d = 3.0$ ,  $T_0 = 2.7$ ; a,  $1 + z_t = 100$  and (from the bottom)  $qZ = 2 \times 10^{-6}$ ,  $2 \times 10^{-5}$ ,  $2 \times 10^{-4}$ ; b,  $1 + z_t = 200$  and (from the bottom)  $qZ = 5 \times 10^{-6}$ ,  $10^{-5}$ ,  $2 \times 10^{-5}$ ,  $5 \times 10^{-5}$ ,  $10^{-4}$  and  $2 \times 10^{-4}$ ; c,  $1 + z_t = 500$  and (from the bottom)  $qZ = 10^{-5}$ ,  $2 \times 10^{-5}$ ,  $5 \times 10^{-5}$ .



could provide an important constraint for this type of model. The ideas presented here can be tested by accurate measurements of the background spectrum both near the peak and at longer wavelengths. Improved measurements at wavelengths  $\geq 20$  cm would be a good test of models which seek to explain the whole background as thermalisation by dust or molecules (and also, incidentally, of distortion due to heat input at very early

pre-recombination epochs). We are working on the exact solution of equations (5) and (6) to check assumption (9). The preliminary result is that equation (9) is valid except for redshifts very close to  $z_1$ .

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## Progressive accretion in the Middle America Trench, Southern Mexico

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*The lithology and age distribution of a seismic unit characterised by landward dipping reflectors suggest progressive underthrusting and uplift of trench deposits along the inner slope of the Middle America Trench. The fastest uplift and deformation rates, and the most rapid change in tilt of dipping reflectors coincide near the base of the trench slope and diminish rapidly landwards.*

PLATE tectonic theory as well as observations at convergent margins have produced several models of trench slopes each making specific predictions as to the origin, age sequence, and structural configuration of the rocks incorporated therein<sup>1-8</sup>. Off-scraped deep-sea rocks arranged in an imbricate stack of landward dipping thrust slices constitute a popular trench-slope model<sup>1,2</sup> which has been applied widely to both modern subduction zones and their supposed ancient equivalents. This imbricate thrusting model is based primarily on seismic reflection data<sup>2</sup> but is poorly substantiated by drilling.

Thick slope aprons and deep water have hindered sampling of inferred off-scraped deposits by drilling in the Marianas and Japan trenches<sup>6,7,9</sup>. Recovery of inferred off-scraped deposits elsewhere has been limited to restricted areas along a given trench slope and has not provided the data necessary to con-

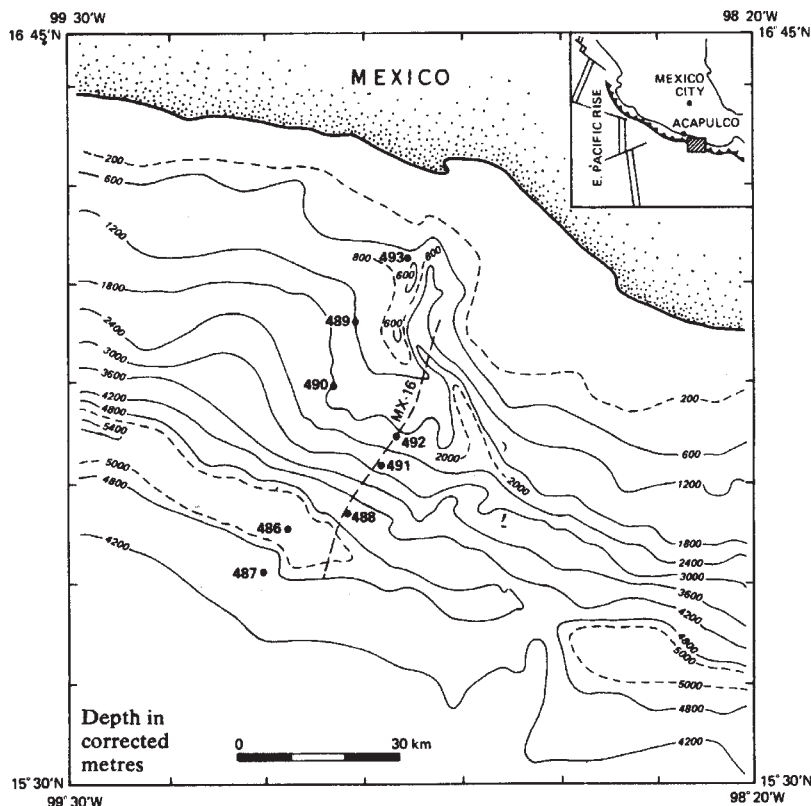
strain models of trench-slope evolution<sup>10-12</sup>. Off southern Mexico the lower slope of the Middle America Trench is covered by a thin slope apron overlying a zone of landward dipping reflectors<sup>13</sup> similar to those which elsewhere are considered to constitute off-scraped deep-sea deposits. In this area, the shallow trench and a thin slope apron permitted drilling of three holes into the zone of landward dipping reflectors which provided substantial data on trench-slope evolution. The drilling was conducted during Leg 66 of the Deep Sea Drilling Project (DSDP).

In the Leg 66 area (Fig. 1) the Middle America Trench is characterised by a narrow shelf, steep inner slope and no forearc basin. Our drilling results confirm previous magnetic<sup>14</sup> and seismic reflection<sup>15</sup> studies indicating that the Mesozoic to Precambrian crystalline basement approaches within 35 km of the trench axis which severely limits the width and volume of off-scraped material. This limited accretionary zone probably developed during the Neogene synchronously with magmatism in the Trans-Mexican belt to the north-east. A more extensive Mesozoic and Palaeogene forearc and accreted zone may have been previously removed<sup>14</sup>.

### Leg 66 drilling results

On Leg 66 DSDP we drilled eight sites (Fig. 1) five of which (Sites 486-488, 491, and 492) are specifically relevant to trench-slope models.

**Fig. 1** Location of Leg 66 drilling sites. Dashed line covers the extent of the seismic profile MX16 shown in Fig. 3. Note the prominent submarine canyon which provides a conduit for transporting coarse-grained detritus directly into the trench and bypassing the slope on which Sites 488, 491 and 492 were drilled.



Sites 486 and 487 were located in the trench and on its outer slope respectively. These sites penetrated trench deposits and sediments overlying oceanic crust thereby sampling the type of material entering the subduction zone and providing a basis for interpreting deposits cored from the trench slope. At site 487 seaward of the trench, we penetrated 115 m of Pleistocene and latest Pliocene hemipelagic mud overlying 55 m of Pliocene to late Miocene brown clay, bottoming in basalt. At Site 486 two holes were drilled on the trench floor separated by ~1 km. In each case the abundance of sand caused the hole to collapse. The thickest section cored at Site 486 consisted of 38 m of fine to very coarse sand with mud. Piston cores taken by the University of Texas Marine Sciences Institute (UTMSI) elsewhere in the trench recovered abundant sand<sup>15</sup> suggesting that this is the dominant lithology in at least the uppermost deposits of the modern trench. Conversely UTMSI cores from the slope apron and lower slope basins in the Leg 66 area contain mud with rare fine thin sand and silt beds<sup>15</sup>. The high content of quartz and feldspar in the sand from the trench suggests it was derived from the crystalline basement of the adjacent continental margin probably via a prominent submarine canyon<sup>16</sup> (Fig. 1). Although the sand in the cores at Site 486 is massive, diagnostic sedimentary structures in some of the UTMSI piston cores from the trench indicates deposition by turbidity currents. Apparently the coarse sand cored at Site 486 moved across the narrow continental shelf and down the submarine canyon to the trench as a density underflow and effectively bypassed the slope.

Sites 488, 491, and 492 penetrated the lower to mid-slope region along UTMSI seismic line MX-16 (Fig. 1) and provided critical evidence for evaluating trench-slope evolution. Site 488, located at the toe of the trench slope at a water depth of 4,254 m, penetrated 313 m of Quaternary mud which below 170 m sub-bottom contains local thin silt and muddy sand beds (Fig. 2). Underlying the mud we cored 115 m of Quaternary mudstone and sand to pebbly sand bottoming at 428 m below the sea floor.

The sand-bearing unit of Site 488 must have been deposited in a major turbidite channel or basin. This unit could have accumulated in a turbidite channel or basin on the slope, however, such a structure was not detected in an exhaustive site survey and would have to have been obliterated. In view of the similarity of sand-bearing unit to deposits cored in the trench,

we prefer to interpret it as an uplifted trench sequence. The virtual absence of significant sand beds in the upper Quaternary slope and slope-basin sediments from the piston and drill cores in the Leg 66 area, and the probable topographic diversion of down-slope sand transport from the site (Fig. 1) both argue against deposition of abundant coarse sand on the slope. If the sand-bearing unit accumulated in a trench of comparable depth to the present trench, then these sediments have been uplifted at a rate of 400–500 m Myr<sup>-1</sup>.

The uniform hemipelagic muds above 170 m at Site 488 probably were deposited near their present position on the lower slope. The thin silt and sand beds between 170 and 313 m require a nearby conduit intermittently supplying fine sand and silt. These thin silt and sand beds probably accumulated when the underlying sand and mudstone unit was slightly elevated above the trench floor but still receiving fine-grained turbidites from the upper portions of large density underflows in the trench axis.

Site 491 lies at a water depth of 2,883 m, ~2 km above and 14 km landward of the Middle America Trench (Figs 1 and 3). Here, we cored 58 m of mud, overlying 380 m of mud containing local thin, fine sand layers, on top of 104 m of sand and pebbly sand interbedded with mudstone of early Pliocene age (Fig. 2). Below 39 m sub-bottom (~2 Myr BP) the sediments are barren of planktonic foraminifera and the nannofossil assemblages are much less abundant suggesting the site passed through the calcite compensation depth (CCD) at this time. In the Leg 66 area surface samples from piston cores and the DSDP sites place the modern CCD at 3,000–3,500 m below sea level. The CCD at 2 Myr BP is placed at 2,750–3,250 m with the shallowing in depth inferred from the documented time-depth variations in the central Pacific Ocean<sup>17</sup>. The depth of the CCD at Site 492 for 4 Myr BP is estimated similarly. We interpret the depositional environments of Site 491 as at Site 488, in terms of the modern example; the sand, pebbly sand and mudstone probably represent trench deposits and the overlying fine-grained sands, silts and muds probably accumulated on the slope during uplift of the underlying sediments. Alternatively, but less likely, the sand, pebbly sand, and mudstone could have accumulated in a lower slope basin or turbidite channel. If the sand, pebbly sand and mudstone accumulated in the trench or on the lowermost



slope at water depths of 4–5 km it was uplifted initially at a rate of about  $400 \text{ m Myr}^{-1}$ . The uplift rate apparently slowed to less than  $100 \text{ m Myr}^{-1}$  after 2 Myr BP when the site passed through the local CCD at a depth of about 3,000 m.

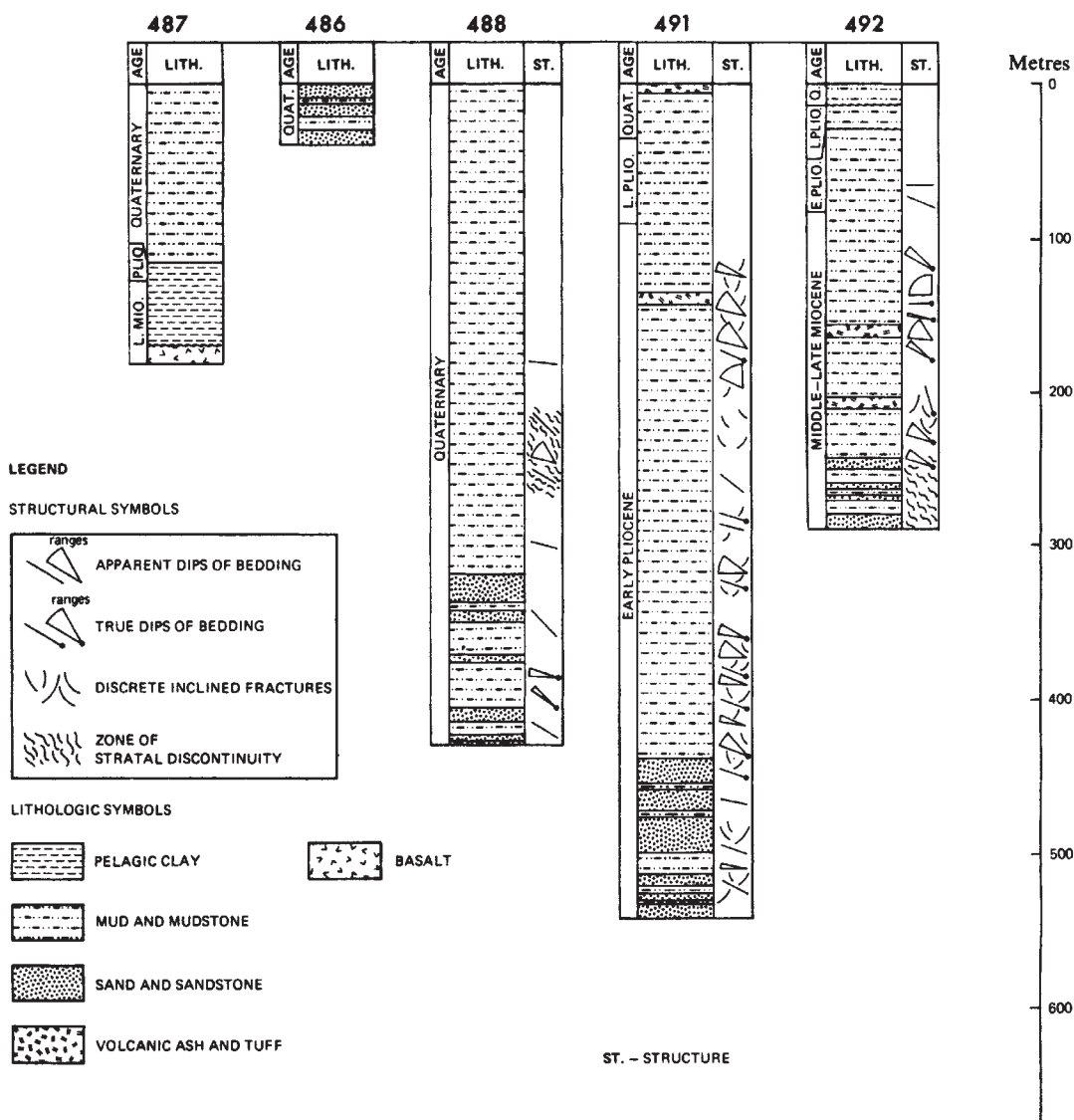
At Site 492 in a water depth of 1,942 m we penetrated 247 m of Quaternary to Upper Miocene mud and mudstone overlying 43 m of Upper Miocene mudstone with sand and granular sand. In sediments about 4 Myr old at 50 m subbottom an abrupt decrease in carbonate content down-hole suggests the site passed through the CCD which is estimated to have been between 2,500 and 3,000 m below sea level at this time. Below 120 m the sediments are barren of foraminifera and contain a *Teichichnus-Zoophycos-Chondrites* trace fossil assemblage suggesting accumulation at depths of 4 km or greater<sup>18</sup>. The sand bearing unit was probably deposited in the trench, based on the lines of reasoning for similar sand sequences at Sites 488 and 491. The age and depth of origin of the trace fossil assemblages, and the time of passage of Site 492 through the CCD define an initial uplift rate of about  $500 \text{ m Myr}^{-1}$  decreasing to about  $200 \text{ m Myr}^{-1}$  after 4 Myr BP.

Sporadic occurrences of shallowly dipping beds usually constitute the initial downhole evidence of deformation at Leg 66 drilling sites. At greater depths a gradual transition to cores with repeated occurrences of dips  $>25^\circ$  represents the threshold

of what we define as significant deformation. In addition to tilted bedding, discrete inclined fractures were common and small scale folds and overturned beds occurred locally. At Site 488 sediments between 210 and 248 m are characterised by healed fractures that truncate bedding and produce zones of stratal discontinuity, evidenced by discontinuous silt lenses in a mud matrix. At Site 492 mudstone intervals below 250 m resemble scaley clay where they are cut by slickensided anastomosing fractures. Deformation begins at shallower depths below the modern sea floor but in older sediments successively landward through Sites 488, 491 and 492 (Fig. 2); the age of the threshold of significant deformation is 0.6, 3.0 and 5.8 Myr at Sites 488, 491 and 492 respectively. Slope sediments are involved in the deformation in all cases; at Site 492 sediments that accumulated at depths as shallow as 3–4 km are deformed significantly.

### Seismic reflection data

An extensive grid of multichannel seismic reflection profiles in the Leg 66 area facilitated site selection and aided interpretation<sup>13</sup>. Figure 3 is a migrated depth-section through Sites 488, 491 and 492, and shows the oceanic basement reflector extending about 15 km beneath the trench slope. Reflections defining the trench fill seem to be acoustically transitional to the zone of landward dipping reflectors underlying the trench slope.



**Fig. 2** Summary of lithology and structural geology for Sites 486–488, 491 and 492. The sand-bearing units, encountered at 313, 438, and 247 m at sites 488, 491 and 492 respectively, correlate with the zone of landward dipping reflectors whereas the overlying mud-rich units correlate with the slope apron (Fig. 3). The depth of significant deformation (repeated occurrences of dips greater than  $25^\circ$  occurs at 220, 116, and 80 m at Sites 488, 491 and 492 respectively.

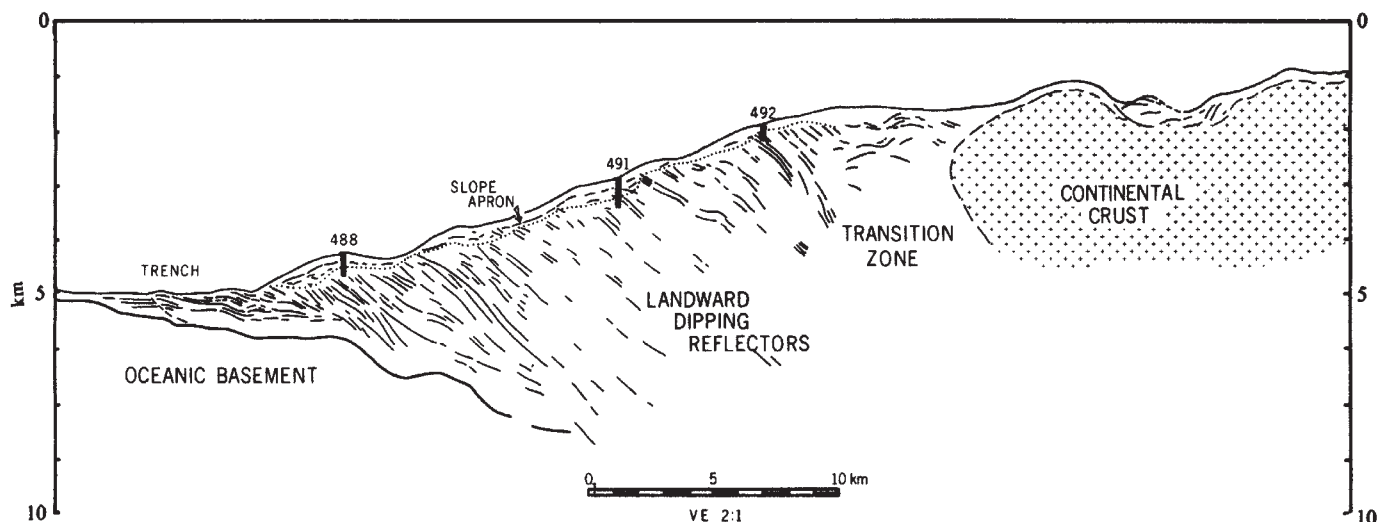


Fig. 3 Line drawing of the depth section MX16. Dotted line separates the slope apron from the landward dipping reflectors. The gas hydrate reflector is omitted for clarity.

Progressive landward inclination of dipping reflectors begins in the lowermost slope area; upslope their inclination remains relatively uniform. The zone of dipping reflectors is overlain by a mantle of discontinuous and indistinct reflectors, the slope apron, with a maximum thickness of 500 m. This slope apron consists of reflectors parallel to both the slope and to the underlying landward dipping reflectors.

The mud-rich sediments comprising the upper portion of the section at Sites 488, 491 and 492, the inferred slope deposits, correlate with the mantle of discontinuous and indistinct reflectors. The zone of landward dipping reflectors correlates with interbedded sand and mudstone units which begin at depths of 313, 438 and 247 m respectively at Sites 488, 491 and 492. Thus many of the landward dipping reflectors here are probably generated not by faults but changes in physical properties across bedding surfaces between the sand and mudstone units. The acoustic similarity of the trench fill and the landward dipping reflectors<sup>13</sup> also suggests that the latter represent bedding surfaces and that the interpretation of the interbedded sands and mudstones as trench deposits is correct.

## Conclusions and discussion

The landward increase in age of the zone of landward dipping reflectors constitutes one of the most fundamental results of drilling on Leg 66 (Fig. 4). Such a systematic age progression indicates seaward growth of the continental margin irrespective of how the dipping reflectors are interpreted. We believe that these landward dipping reflectors are bedded units generated by thick sand and mudstone sequences which probably accumulated in the trench, or less likely on the lower slope. Thus, the landward increase in age in these dipping reflectors represents a stratigraphic inversion most simply explained by underthrusting along surfaces sub-parallel to bedding.

Shipboard palaeontological results did not resolve stratigraphic inversions in a single drillhole, even though one site (Site 488) was located to penetrate a seismically inferred thrust fault. Nevertheless, zones of stratal discontinuity and scaley clay, and anomalies in physical properties, suggest faulting and/or shear zones cutting the section cored at Sites 488 and 492. The gross stratigraphic inversion between sites suggests that thrusting occurs within discrete zones between our drilling locations and/or as distributed shear on numerous faults of small displacement. The pervasively sheared and disrupted fabrics common to inferred ancient and subduction complexes support the latter interpretation.

Although our palaeobathymetric data are preliminary, they consistently indicate uplift of the trench slope at Sites 488, 491

and 492. Quaternary rates are most confidently established suggesting uplift of 400–500 m Myr<sup>-1</sup> at Site 488, decreasing to 200 m Myr<sup>-1</sup> or less at Sites 491 and 492. Significantly, palaeobathymetric data suggest that the inferred trench and/or lowermost slope deposits of Sites 491 and 492 underwent rapid uplift which subsequently decreased to the slower rates characteristic of the Quaternary. Thus both the present distribution of Quaternary uplift rates and the historical records of Sites 491 and 492 argue for a high rate of uplift on the lowermost slope with a diminishing rate of uplift landwards.

The deformation in cores from Sites 488, 491 and 492 includes overturned folds, small-scale disruption of layering and scaley clay all of which occur commonly in subaerially exposed subduction complexes. In the Leg 66 cores the trench and the immediately overlying lowermost slope sediments are similarly deformed though the intensity of deformation decreases upsection through sediments accumulated at shallower depths. The tilting of sedimentary layering in cores accumulated at 3–4 km of water depth (50–120 m sub-bottom) at Site 492 indicates continuing deformation of the slope a substantial distance from the trench. Thus since the deformation is

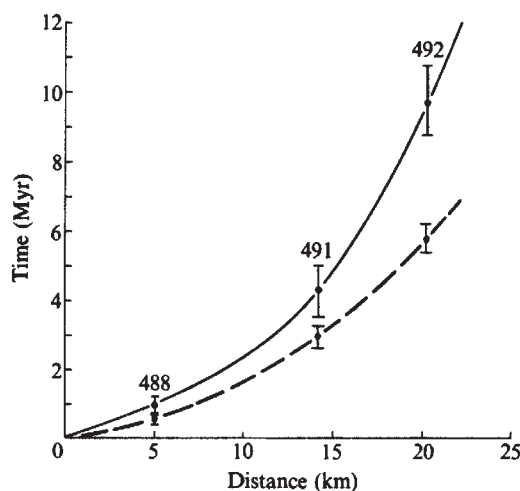


Fig. 4 Age of dipping reflectors (solid line) and youngest significant deformation (dashed line) versus distance from base of trench slope. Age of dipping reflectors taken from age of top of sand-bearing units (Fig. 2). Age at which youngest significant deformation occurs specified in caption, Fig. 2. Error bars indicate uncertainty in biostratigraphic age determinations.



apparently continuing upslope, away from the trench, the age of significant deformation at each site (Fig. 4) can be used as an indicator of the relative rates of deformation. The time required to develop significant deformation increases by a factor of 5 from 5 to 14 km from the base of the slope, and by an order of magnitude from 5 to 20 km from the base of the slope (Fig. 4). In other words, the rates of deformation at Sites 491 and 492 are respectively one-fifth and one-tenth of that observed at Site 488.

The folded beds and disrupted layering documented at Sites 488, 491 and 492 are probably due to both tectonic and gravity processes; the evidence concerning individual structural fea-

tures is ambiguous. Nevertheless, correlation of the high rate of deformation at Site 488 with the high rate of uplift and rapid change in tilt of dipping reflectors indicates a concentration of tectonism near the base of the trench slope. Thus if gravitational processes are active in producing the structural features seen in the cores they too are accentuated at the base of the trench slope probably in response to the concentration of tectonic activity.

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# A volcanic ash generated by explosions where ignimbrite entered the sea

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*A remarkably widely dispersed and crystal-rich volcanic ash-fall deposit is described, which resulted from immense explosions generated in the littoral zone in New Zealand where a major rhyolitic ignimbrite flowed into the sea. This partially explains what happens when large pyroclastic flows enter water.*

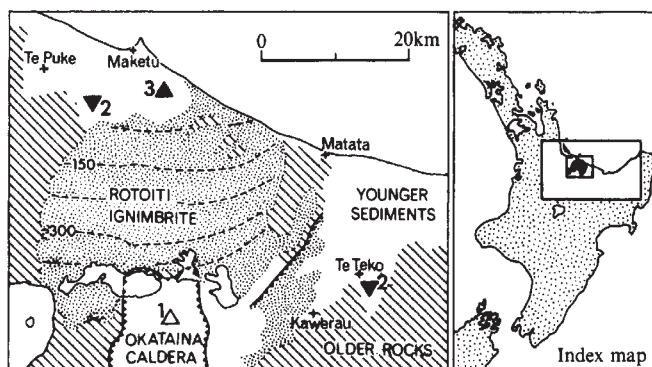
THE rhyolitic volcanoes of the Taupo Zone have produced extensive air-fall ashes in addition to the ignimbrites for which they are better known. The tephrochronology of these ashes is well documented<sup>1–4</sup>, but volcanological interpretations have seldom been attempted. The greatest air-fall deposit of the past ~50,000 yr is the Rotoehu ash, part of which is described and interpreted here.

The Rotoehu ash is remarkable for its large volume, previously estimated<sup>5</sup> to be at least 50 km<sup>3</sup> but now believed to be much more. It is also widely dispersed over most of the North Island of New Zealand, and is extremely rich in crystals which show up in its coarser beds. An isopach map is given by Vucetich and Pullar<sup>5</sup>. It is a distinctively shower-bedded fall deposit, and here we consider the thickest and uppermost and most widely dispersed of the three coarsest beds (bed G in Fig. 2).

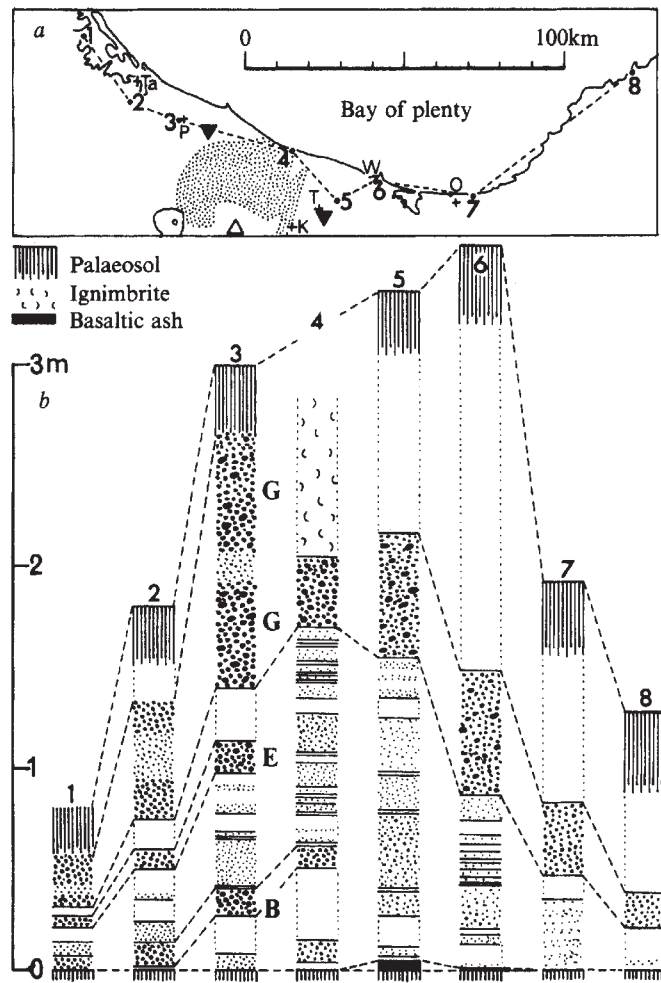
The Rotoehu ash is intimately associated with the non-welded Rotoiti ignimbrite, the latest major ignimbrite from the Okataina volcano, and Nairn<sup>6</sup> has demonstrated that parts of the ash underlie, overlie, and are interbedded with flow units of the ignimbrite. The ignimbrite covers about 830 km<sup>2</sup>, Fig. 1, and is known by its stratigraphic name as the Rotoiti Breccia. It is believed to have originated from a vent or vents in the Haroharo complex of Okataina volcano, and reached the coast of the Bay of Plenty 30 km from source along a front of more than 10 km. The <sup>14</sup>C age is about 42,000 years<sup>7,8</sup>. The estimated volume is 50 km<sup>3</sup>.

## Characteristics of the deposit

Bed G has a loose and sandy texture, and within 50 km of the Rotoiti ignimbrite outcrop it contains scattered fragments of ragged pumice up to several centimetres in size. It is conspicuously rich in crystals, resembling a waterlain sand. The relative uniformity in grain-size and thickness over a great area, the perfect mantle bedding, and the lack of rounding of the grains,



**Fig. 1** Map showing the main outcrop of the Rotoiti ignimbrite, and the approximate locations of its source vent (1), the two littoral vents of Rotoehu ash bed G (2), and the littoral vent of ash bed E (3). The ignimbrite forms a northward-sloping aggradational surface (dashed lines indicate generalised contours, heights in metres); anomalously high south of Matata which is attributed to post-Rotoiti uplift of the land bordering the young rift valley in which Te Teko is situated. An older non-welded ignimbrite separated from the Rotoiti by a soil also occurs but is not distinguished on the map. The extent of the Rotoiti ignimbrite under the sediment flats of Te Puke and Te Teko is not known. The index map of the North Island of New Zealand shows the location of the studied area.



**Fig. 2** *b*, Profiles of the Rotoehu ash at locations shown on the sketch map (*a*), in which the grain size of the beds is shown by the coarseness of the stipple (the finest ash is unstippled), showing the relative positions and thickness variations in the three main sandy beds, B, E and G. Place names K, Kawerau; O, Opotiki; P, Te Puke; Ta, Tauranga; T, Te Teko; W, Whakatane.

however, leave no doubt as to its shower origin. In the east and south, G is the coarsest and most prominent sandy bed in the Rotoehu ash. It is underlain by well bedded fine and sandy ashes, and is overlain by fine loess-like ash. In the west, G is the thickest, though not always the coarsest, of the sandy beds; it has a rather finer middle part and grades upwards to finer ash, and the weathered zone often extends well down into it, destroying its loose sandy texture.

The maps of bed G, Fig. 3, reveal the wide dispersal; the thickness and maximum grain-sizes decrease to half their value over a distance of 20–50 km, which is a remarkably low decrease rate for a fall deposit.

Sieve analyses have been made of 54 samples of G, using a set of sieves in which the aperture size of each is half that of the sieve above, and for each sample the percentages of components (pumice, free crystals, and lithics) have been determined for each size class down to 0.25 mm. For convenience, the size class is denoted by the aperture size of the sieve that retains it. 'Pumice' includes vesiculated juvenile fragments whatever their size, and also the bubble-wall shards which are abundant in fractions finer than 0.25 mm.

Lateral variations in grain-size characteristics are shown in Fig. 4*a*, and in crystal content by Figs 4*b* and 5. Figure 4*c* shows that the percentage of crystals decreases outwards in each size class due to aeolian fractionation: crystals have fallen closer to

source than pumice of the same size. The decrease rate is remarkably low: crystals constitute more than 50% of the 0.5 mm class out to as far as 80 km from the ignimbrite outcrop.

South of Rotorua, G is missing and ash rich in accretionary lapilli occurs instead. Fine ash was thought to be flushed by rain from the eruptive cloud together with coarser material near Rotorua so that bed G is not recognisable as such, and by the time the cloud spread farther south it had largely been cleansed of ash.

## Origin

The thickness and maximum grain-size decrease rate resembles that of a plinian pumice, but bed G differs from such a deposit in three ways. First, it totally lacks ballistic clasts; the largest pumice and lithic fragments noted measure 8.4 and 4.6 cm in maximum size respectively. Second, the median diameter is never coarser than 1 mm. Third, the overall grain-size distribution clearly has a strong mode in the 0.5 and 0.25 mm classes which predominate in all samples out to 100 km from the ignimbrite, Fig. 4*a*, and this mode consists mainly of crystals.

Bed G is interpreted as originating from vast explosions at two places where pumice flows of the Rotoiti ignimbrite entered the sea, based on two independent lines of evidence: the field data, and the peculiar grain-size characteristics of the bed.

First, the thickness and maximum grain-sizes reach a maximum at two places about 45 km apart, one near Te Puke and the other near Te Teko, Fig. 3 *a,c,d*, and likewise the content of coarse material from sieve analyses, Fig. 3*e*. These sites are on alluvium and are believed to mark the rootless vents of the littoral explosions by which bed G was generated. Sea bays are postulated to have projected to them at the time of the eruption. The thickness and grain-size decrease rate out from the Te Teko vent is lower than from the Te Puke vent suggesting that the Te Teko explosion was the more powerful, and the lack of stratification in bed G in the eastern area indicates a single short explosion of uniform intensity.

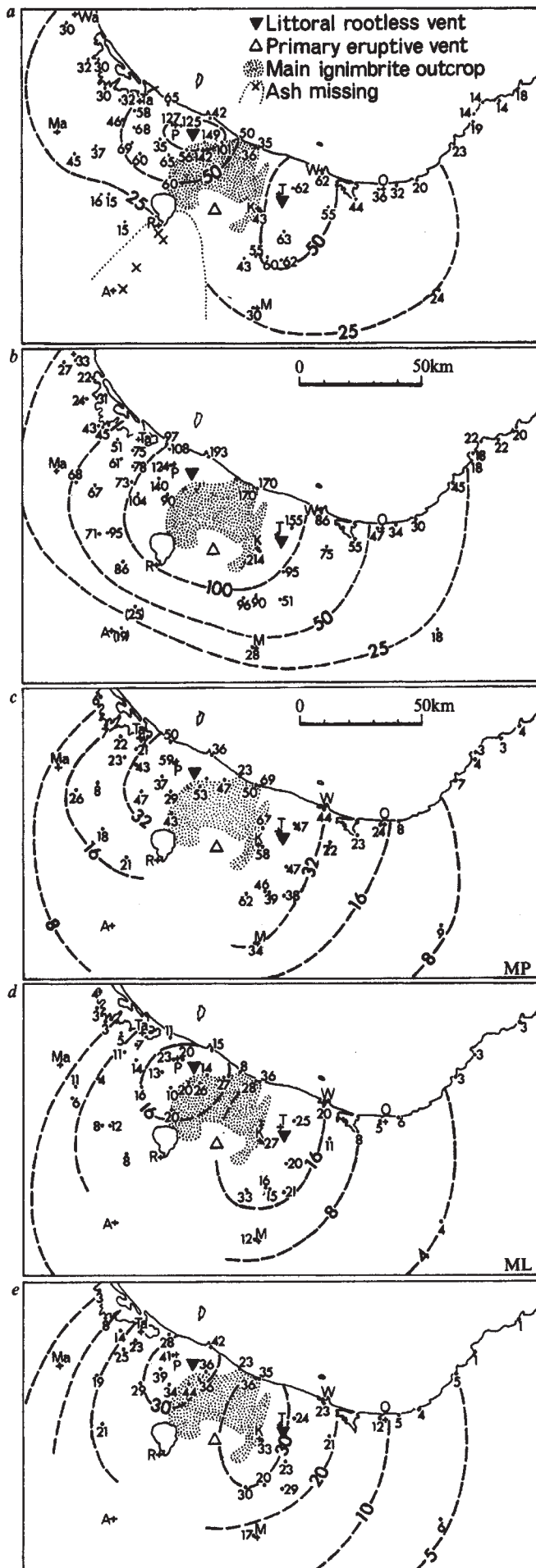
Second, G originated from a pyroclastic mixture that was more like that of a pyroclastic flow than that ejected at a primary explosion vent. Consider the special grain-size features of ignimbrite and their origin. Ignimbrite is constituted from the pyroclastic mixture which leaves the primary vent. The crystals are phenocrysts liberated from the magma, and the phenocryst content of large pumice clasts taken from the ignimbrite is believed to equal that in the original magma and for the Rotoiti ignimbrite and bed G is 24 wt %.

Three processes then modify the pyroclastic mixture so that the resulting ignimbrite departs considerably from the initial population. One is a loss of fine vitric particles, from the eruptive column and the moving flow, which eventually settle as a co-ignimbrite ash-fall largely beyond the limits of the ignimbrite. The loss generally amounts to about half of the total erupted mass, as is indicated by the two-fold concentration of free crystals in the ignimbrite<sup>9,10</sup>. The second process is the settling out of heavy clasts from the pyroclastic flow, the largest as a near-vent lag-fall deposit<sup>11</sup>, and smaller ones as a lithic concentration zone near the base<sup>12</sup>, or as a ground layer beneath, the ignimbrite<sup>13</sup>. This results in an outward decrease in maximum lithic size and content<sup>14</sup>. The third process is a progressive fragmentation of pumice within the flow.

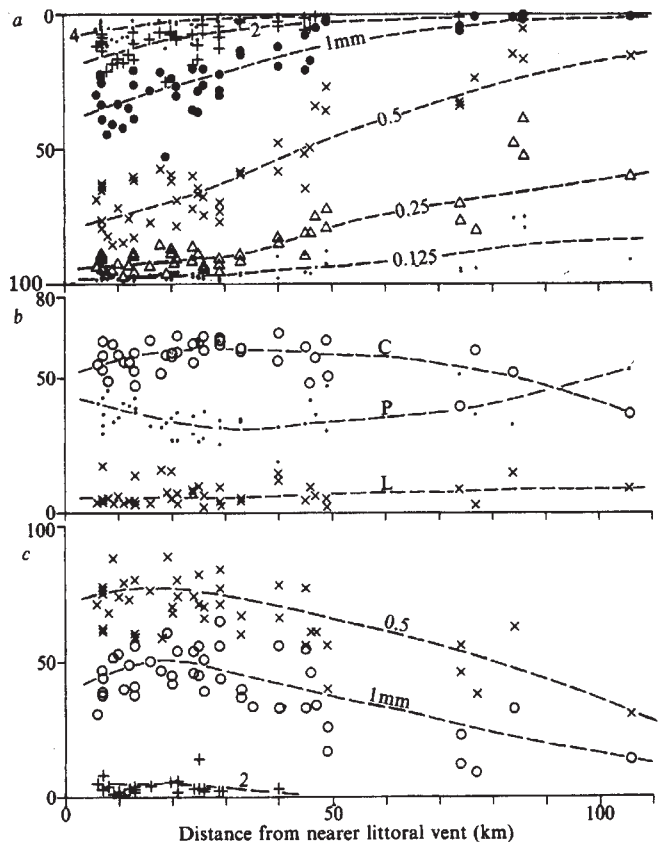
All three processes seem to have operated in the Rotoiti ignimbrite. In distal exposures the ignimbrite has a low content of lithics and a maximum lithic size of ~5 cm; the content of free crystals is high, with a strong mode in the 0.5 and 0.25 mm classes; and the pumice distribution is strongly fine-skewed.

The characteristics of the distal Rotoiti ignimbrite are summarised by Fig. 6*a*. A big contrast exists between samples taken from near the base or from the middle/upper part, the former being much richer in lithics and the latter in coarse pumice (Fig. 5), and Fig. 6*a* is averaged from three samples taken from each position. The ignimbrite consists of many flow units, and the one which gave rise to bed G has not been identified.





**Fig. 3** Dispersal maps of the Rotoehu ash bed G. ▼, The rootless vents of Te Puke (P) and Te Teko (T). a, Thickness (cm); b, thickness of the sub-G part of the Rotoehu ash (cm); c, average maximum diameter of the three largest pumice clasts (mm); d, average maximum diameter of the three largest lithic clasts (mm); e, wt % of material coarser than 1 mm. Place names as Fig. 2, with A, Atiamuri; Ma, Matamata; M, Murupara; R, Rotorua; Wa, Waihi.



**Fig. 4** Characteristics of the Rotoehu ash bed G as they vary with distance from the nearer littoral vent: a Cumulative wt% coarser than the size stated, for all sieved samples. Note the broadness of the band between 1 and 0.25 mm; the 0.5 and 0.25 mm size classes predominate in all samples irrespective of their distance from the vent. b, wt% of pumice (P), crystals (C) and lithics (L) in the sum of the 0.25 and coarser classes, recalculated to 100% total. Note that crystals predominate in almost all samples; c, The wt% of crystals in three grain-size classes, expressed as a percentage of that class.

The overall grain-size constitution of G has been partially determined as for the Taupo plinian pumice<sup>15</sup>, by drawing maps of the mass per cm<sup>2</sup> of each size class and then integrating to yield the total mass of the class. These integrated values are plotted in Fig. 6b and compare closely with the average Rotoiti ignimbrite. The origin of bed G from Rotoiti ignimbrite material can be deduced from the size data and constitution since these are predetermined by the pyroclastic mixture available, characterised by a small maximum clast size, a high content of crystals, and a small content of pumice coarser than 1 mm.

Phreatomagmatic pyroclastic deposits formed where water enters the primary vent have a similar strongly fine-skewed population<sup>16</sup>, but ballistic fragments are also invariably present. Furthermore, such eruptions are pulsatory, so that fine material from weaker activity falls together with coarse from stronger activity, to produce poorly sorted deposits even very close to vent; in contrast, bed G is well sorted everywhere.

The volume of bed G within the 25 cm isopach is 6 km<sup>3</sup>, assuming bilateral symmetry about the Te Puke-Te Teko line, but much lies outside this isopach. When a plot of the area

enclosed by isopachs is extrapolated out to 1 cm, the volume by integration is  $20 \text{ km}^3$ , but the extrapolation is uncertain. The formula of Cole and Stephenson<sup>17,18</sup> (taking the two vents separately) yields a total of  $14 \text{ km}^3$ .

Another way of estimating the volume is by assuming that G originated from the average Rotoiti ignimbrite of Fig. 6a. Based on the quantity of material 0.5 mm or coarser (Fig. 6b), the total mass of G is  $12 \times 10^{15} \text{ g}$  (equals  $11 \text{ km}^3$  at a density of  $1.07 \text{ g cm}^{-3}$ ); based on the content of crystals 0.5 mm or coarser, the mass is  $18.6 \times 10^{15} \text{ g}$  ( $=17 \text{ km}^3$ ); and based on the quantity of crystals in the 0.5 mm class the mass is  $20.5 \times 10^{15} \text{ g}$  ( $=19 \text{ km}^3$ ).

## Discussion

The exceptionally wide dispersal of bed G, and the efficient aeolian fractionation of crystals even 100 km from source, indicate that the secondary eruptive columns above the littoral explosions must have been exceedingly powerful. The area enclosed by selected maximum pumice and maximum lithic size isopleths (Table 1) is comparable with that given by the most powerful known plinian eruption, that of Taupo AD 131 (ref. 15). It is very much greater than for other known deposits of rootless vents (for example, in Iceland<sup>19</sup> and Hawaii<sup>20</sup>).

The extreme violence of the Rotoehu event must result from an exceedingly high energy release rate. The best mechanism seems to be one in which about  $15 \text{ km}^3$  of hot pyroclastic flow entered the sea very rapidly at Te Puke and Te Teko and spread over perhaps tens of square kilometres before coming to rest. The first part to enter the sea became well mixed with water, and the rapid exposure of a great surface area of the hot particulate material resulted in a very rapid heat exchange with the water. This part, however, was rapidly over-ridden and buried beneath newly-arrived material, delaying the explosive expansion of the resulting steam. When the supply of material fell, the pressure build-up exceeded the rate of addition of material, and explosive disruption of the entire mass then occurred.

The abnormally high crystal content of bed G stems from an initial high magmatic content, combined with the subsequent successive operation of two crystal concentration processes: the selective loss of vitric dust above the primary vent and moving flow, and the aeolian fractionation of material falling from the

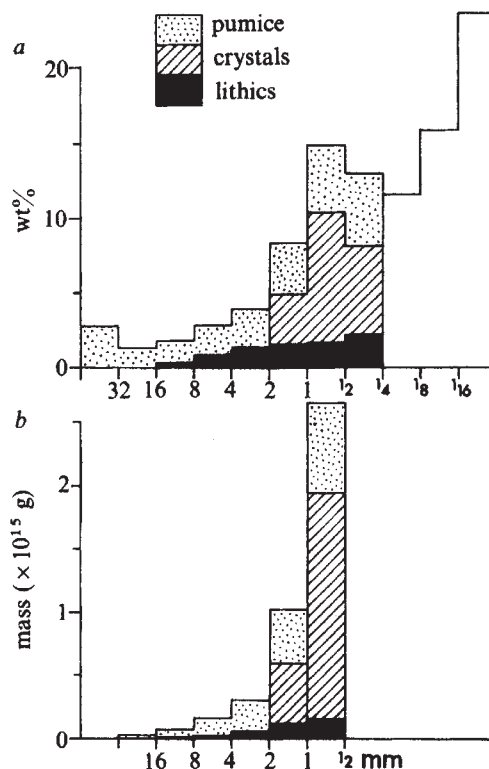


Fig. 6 a, Histogram of the average grain-size distribution in six sieved samples of the Rotoiti ignimbrite; the bars are subdivided on their weight percentages of components. b, Histogram of the total mass of grain-size classes and their components in the mapped part of the Rotoehu ash bed G; the vertical scale has been adjusted so that the total length of bars for the 0.5 mm and coarser classes in (a) and (b) is the same.

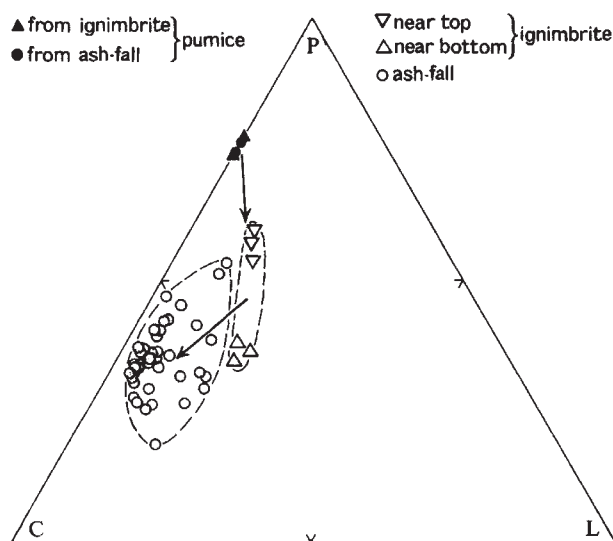


Fig. 5 Triangular plot showing the weight percentages of pumice (P), crystals (C) and lithics (L) in samples of the Rotoehu G ash and Rotoiti ignimbrite, for the sum of the 0.25 mm and coarser classes recalculated to 100% total. Crystal contents of pumice clasts from the ash and ignimbrite were determined by crushing followed by mechanical separation, and are thought to be representative of the original magma. The arrows show the two stages of crystal enrichment beginning with the original porphyritic magma.

littoral explosions. The exceptional violence of the explosion resulted in a high crystal content persisting even to 100 km from source.

The other two main sandy layers (B and E) in the Rotoehu ash have similar characteristics and formed by similar explosions of earlier Rotoiti ignimbrite flow units. Bed E was evidently formed where a flow entered the sea south-east of Maketu (3, Fig. 1). Its volume and dispersal are comparable with the eastern part of G from the Te Teko rootless vent. The fine ashes within the Rotoehu ash may be normal co-ignimbrite ash-falls or may represent weaker littoral activity.

The Te Teko explosion which gave rise to half of bed G and that which gave rise to bed E seem to have been the most violent volcanic explosions yet documented by ash dispersal studies. This is reminiscent of the 1883 eruption of Krakatau, which was accompanied by major explosions and culminated in three immense paroxysms during which tsunamis were generated. It has long been supposed that seawater was a major factor in producing such extreme violence, but there has always been uncertainty regarding the mechanism: whether it was by the sea entering the magma chamber or not.

Another possibility is that the Krakatau explosions were indeed caused by the interaction of magma and the sea, but the explosions were littoral ones where pyroclastic flows entered the water as at Te Puke and Te Teko. The classic studies of the pyroclastic products of Krakatau by Verbeek<sup>21</sup> and Stehn<sup>22</sup> indicate that pyroclastic flow deposits on land were formed at the time of the biggest explosions. Testing of this idea, must, however, await a critical re-examination of the pyroclastic products of this event.

The picture which emerges is that ash bed G was deposited from an immense mushroom cloud which was tens of kilometres high and rapidly expanded until it was well over 200 km in diameter. This mushroom spread laterally outwards nearly



**Table 1** Areas enclosed by maximum grain size isopleths ( $\times 10^3 \text{ km}^2$ )

|                              | Maximum pumice<br>(mm) |      |      |     |     |
|------------------------------|------------------------|------|------|-----|-----|
|                              | 64                     | 32   | 16   | 8   | 4   |
| Rotoehu bed G*               | 0.2                    | 3.4  | 14   | 24  | 44  |
| Taupo plinian                | 5                      | 9.9  | 18   | 28† | 40† |
| Pompei plinian <sup>23</sup> | 0.3                    | 0.9  | 1.9† | 3†  | —   |
|                              | Maximum lithic<br>(mm) |      |      |     |     |
|                              | 32                     | 16   | 8    | 4   |     |
| Rotoehu bed G*               | 0.1                    | 2.5  | 12   | 25  |     |
| Taupo plinian                | 2.3                    | 4.2  | 7    | 15  |     |
| Pompei plinian <sup>23</sup> | 0.6                    | 1.1† | 1.6† | —   |     |

\* Assuming a symmetrical distribution on either side of the Te Puke-Te Teko line.

† Extrapolated values.

uniformly in all directions. The material which fell 100 km out from source has a median terminal fall velocity of  $3 \text{ m s}^{-1}$ , for which the fall time from a height of 10–50 km in normal atmosphere is 35–70 minutes. This suggests that the outward radial velocity of the expanding cloud averaged  $180\text{--}90 \text{ km h}^{-1}$  neglecting the dustiness of the atmosphere at the time and turbulence. The lateral expansion was apparently much greater than is normal in volcanic explosions, and was comparable with the most powerful one currently known. In normal explosions,

the expansion is due to magmatic gases which constitute a few wt% of the magma. In the Rotoehu case it is possible that steam constituted a few tens of wt% of magma involved.

This ash is an example of a new class of pyroclastic deposit for which the name 'littoral co-ignimbrite ash-fall' is proposed.

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# Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in *E. coli*

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*The complete nucleotide sequence of hepatitis B virus genome (subtype ayw) cloned in Escherichia coli has been determined using the Maxam and Gilbert method and the dideoxynucleotide method. This sequence is 3,182 nucleotides long. Location of the nonsense codons shows that the coding capacity of the L chain is larger than the coding capacity of the S chain. Eight open regions, able to code for polypeptide chains larger than 100 amino acids, have been located. Region 6, which is the largest, covers more than 80% of the genome. The gene S which codes for polypeptide I of the Hbs Ag and was previously located between coordinates 95.1 and 73.6 is contained in region 7.*

HEPATITIS B VIRUS (HBV) infects man at a very high rate. In western Europe and USA, about 10% of the population carry an HBV serological marker such as the surface antigen (HBs Ag) or the corresponding antibody (HBs Ac)<sup>1</sup>. In some areas of tropical Africa and southeastern Asia, this percentage can be as high as 60% or 70% (ref. 2). Recovery is spontaneous in most cases, but the virus can cause very severe complications like fulminant hepatitis, chronic hepatitis and cirrhosis in addition to which a hepatocarcinoma can develop<sup>3</sup>. It has been estimated that there

are at least 120 million chronic carriers of HBs Ag, the so-called healthy carriers, throughout the world<sup>2</sup>.

In addition to HBs Ag, two other antigens have been identified: HBc Ag which corresponds to the core of the virus, and HBe Ag whose location within the virion has been recently demonstrated<sup>4</sup>. It was also shown that the viral genome is made up of a circular DNA molecule which is partially single-stranded and about 3,200 bases long<sup>5,6</sup>. The virion also harbours a DNA polymerase able to repair the viral DNA<sup>7</sup>. The novel structure of this virus makes its classification somewhat difficult.

The biological study of the virus is complicated by the fact that HBV cannot be propagated in cell cultures and the only available viral source remains human serum. However, cloning of the HBV genome in *Escherichia coli* has allowed the purification of large amounts of genetically homogeneous HBV DNA, and the establishment of several restriction maps<sup>8–10</sup>. Hence we have recently<sup>11</sup> localised and determined the primary structure of the S gene which codes for polypeptide I and its glucosylated derivative polypeptide II, which are the major components of the HBs Ag.

In this article, we report the complete primary structure of the viral genome (subtype ayw) previously cloned in *E. coli*.

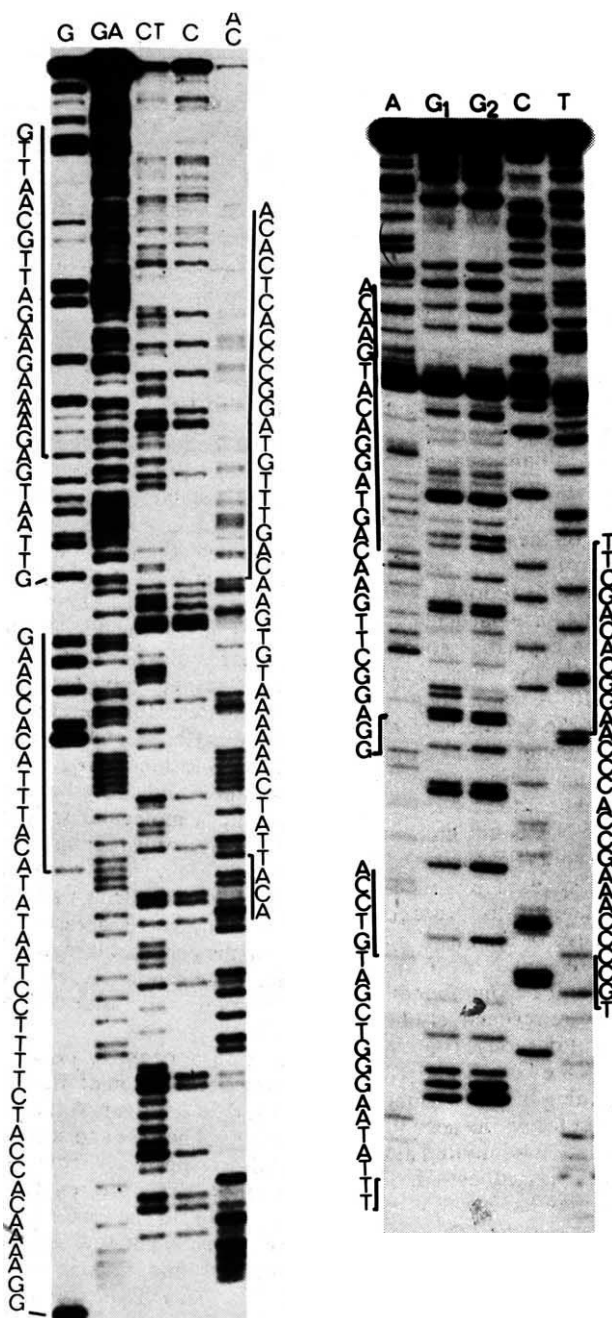
## Primary structure of the cloned viral genome

To determine the nucleotide sequence of this DNA, the chemical degradation method of Maxam and Gilbert<sup>12,13</sup> and the dideoxynucleotide method of Sanger *et al.*<sup>14</sup> as modified for

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**Fig. 1** Viral DNA (serotype ayw), repaired with the endogenous DNA polymerase and the four nucleotide triphosphates, was digested with *EcoRI* endonuclease and cloned in *E. coli* using  $\lambda$  gt WES bacteriophage as the cloning vehicle<sup>16</sup>. The cloned DNA was referred to as *Eco* HBV DNA. To avoid genetic drift, all stocks used to analyse the DNA sequence were made on plates from the same lysate obtained after the first cloning step. Recombinant phages were purified by two successive CsCl gradients and the DNA was phenol extracted<sup>11</sup>. After hydrolysis with *EcoRI* endonuclease, *Eco* HBV DNA was separated from the two  $\lambda$  arms by sucrose gradient ultracentrifugation<sup>17</sup>. To prepare each set of restriction DNA fragments, 25 to 30  $\mu$ g of *Eco* HBV DNA were fully hydrolysed with one of the restriction enzymes described in Fig. 2, using the conditions recommended by the manufacturer (Biolabs). Restriction fragments were dephosphorylated by incubation at 65 °C for 45 min with 4  $\mu$ l of bacterial phosphatase. This enzyme was then inactivated by alkaline treatment<sup>18</sup> and DNA fragments were 5' labelled with [ $\gamma$ -<sup>32</sup>P]ATP (NEN; specific activity 2,500 Ci mmol<sup>-1</sup>) and polynucleotide kinase (P. L. Biochemicals) as described by Maxam and Gilbert<sup>12</sup>. Labelled fragments were fractionated by gel electrophoresis and eluted from the gels as previously described. The two labelled ends of each restriction fragment were then separated either by hydrolysis of the DNA fragment with another restriction endonuclease and gel electrophoresis or by heat denaturation in the presence of 30% dimethyl sulfoxide and gel electrophoresis<sup>13</sup>. DNA fragments labelled at one 5' end were eluted from the preparative gel and dissolved in water. Chemical reaction, specific for G, A + G, C + T, T and A + C giving a stronger band for A than C were performed as described previously<sup>12,13,17</sup>. Sequence reaction products dissolved in loading solution were heat denatured and fractionated on 25, 16 and 8% acrylamide gel. Figure 1a is an example of fractionation on an 8% acrylamide slab gel of the sequence reaction products obtained with *Hinf* C' DNA fragment (nucleotides 658 to 552). When the two labelled ends were separated by hydrolysis with a restriction enzyme, nucleotide sequences were also determined with the dideoxynucleotide method<sup>14</sup> adapted by Maat and Smith<sup>15</sup> for double-stranded DNA fragments. Figure 1b is an example of fractionation on a 16% acrylamide gel of such reaction products obtained with *Bgl*A' fragment (nucleotide sequence from residue 1,264 to residue 1,338). The two G lanes correspond to two different reaction conditions. In G1 there is no dGTP while in G2 the final concentration of dGTP is 2  $\mu$ M. In both reactions, there is 1 mM ddGTP, final concentration. Subscript prime indicates the position of the <sup>32</sup>P on the L strand, subscript second, its position on the S strand.



double-stranded DNA by Maat and Smith<sup>15</sup> were used. Two examples of sequencing gel autoradiograms are shown in Fig. 1. To analyse independently both DNA strands and to overlap all the restriction sites used as starting points, a large number of fragments were analysed (Fig. 2). The localisation of the 5' end of the largest DNA strand on the *Bam*HI viral restriction map and the knowledge of this restriction map on the *Eco* HBV DNA allowed the identification of the cloned DNA strands with respect to the viral DNA strands. Here we call the DNA strand of *Eco* HBV DNA homologous to the longest viral DNA strand the L strand, and the other, the S strand. The sequence shown in Fig. 3 corresponds to the *Eco* HBV DNA, which is 3,182 nucleotides long.

### Location of the open reading frames on the viral genome

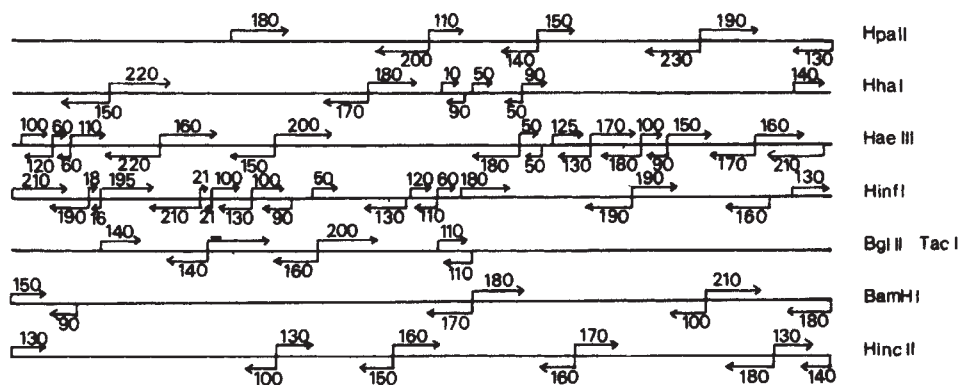
A computer program was used to search for nonsense codons on both DNA strands<sup>19</sup>. It appears from Fig. 4 that the number and distribution of nonsense codons are very different on S and L strands. Considering the total genome length, a random distribution of nucleotides should give about 50 nonsense codons in each reading frame. Instead one observes 33, 29 and 15 nonsense codons in the S strand for each phase and 61, 60 and 41 nonsense codons on the L strand. In addition, their distribution along the DNA molecule is very different on the two strands. On the L strand, the open reading frames are relatively short (Fig. 4). Four regions are able to code for 100 amino acids or more. These four regions labelled 1 to 4 are 510, 327, 447 and 345

nucleotides long. On the S strand (Fig. 4), the open regions are much longer. These regions labelled 5 to 8 are 498, 2,553, 1,296 and 657 nucleotides long. The length of the above hypothetical coding regions which include the *Eco*RI restriction site (such as regions 4, 6 and 7) is only valid if no DNA has been lost in the vicinity of this restriction site on cloning, which is most likely. In spite of this limitation, the distribution of stop codons clearly shows that the coding capacity of the L strand is larger than that of the S strand.

Regions 1, 2, 3 and 4 appear to be located on the genome as two pairs (Fig. 5)—region 3 is located immediately downstream of region 1 and region 2 immediately beyond region 4. Regions 5, 6, 7 and 8 extend over the whole genome and overlap with each other in different reading frames (Fig. 5). Region 6 covers about 80% of the genome and includes the whole of region 7 and part of regions 5 and 8. Regions 6 and 7 correspond partly to the single-stranded region of the genome. Regions 4 and 5 cover the 5' extremity of the S strand and the nick of the L strand.



**Fig. 2** Diagram of analysed DNA fragments. Vertical bars correspond to the position of the 5' labelled ends of restriction fragments used to determine the nucleotide sequence of the *Eco* HBV DNA. Numbers above each arrow indicate the length of nucleotide sequences analysed from the restriction sites.



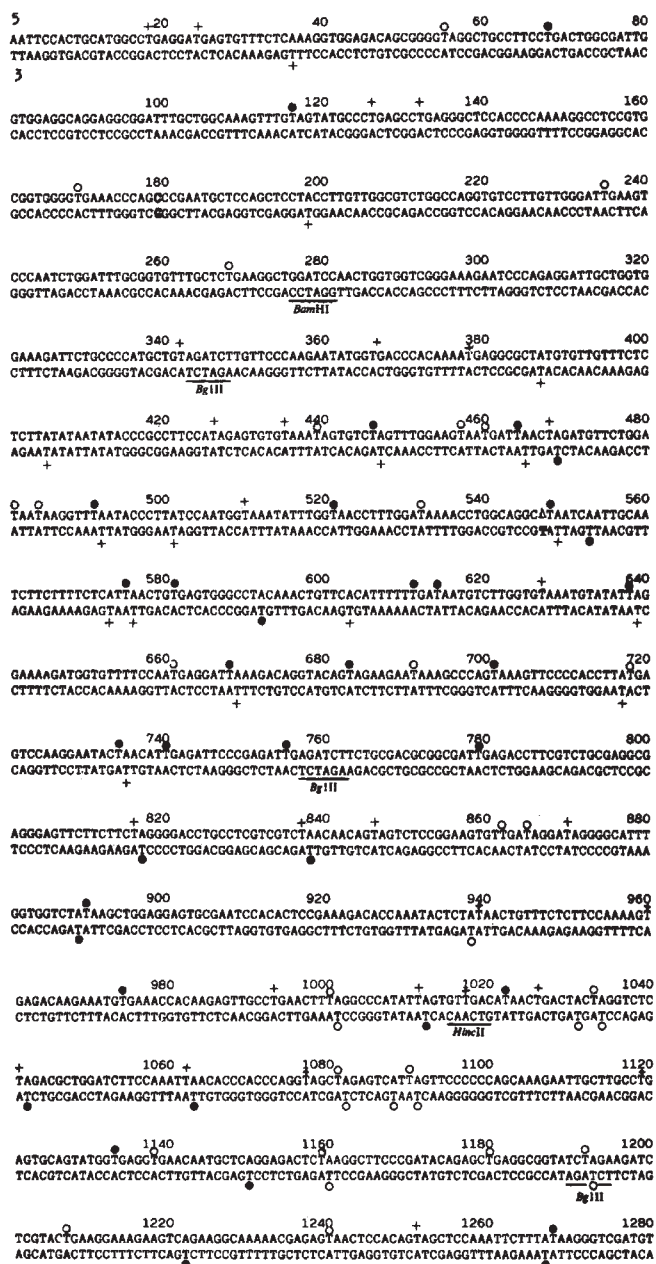
### Tentative translation of open regions

Few proteins have been shown to be coded by the viral genome<sup>9,11</sup>, but one can suppose that, because of its very small size, regions with open reading frames are largely used for translation. Therefore, a search for the ATG codons which could initiate translation has been made near the beginning of these regions, although the existence of mRNA splicing<sup>20,21</sup> could complicate the translation of the open regions. The positions of these ATGs are shown by small arrows in Fig. 5. In region 1 to 4, the numbers of codons located downstream from these ATG codons are 79, 60, 145 and 28 respectively. Regions 2 and 4 have particularly low coding capacities. However, these coding capacities could be modified by mRNA splicing. For example, the translation from the first ATG located in region 4, of mRNA transcribed from regions 4 and 2 and spliced could allow the synthesis of a polypeptide with a molecular weight (MW) up to 18,000. For simplicity and clarity, only regions with an open reading frame able to code for 100 amino acids or more are taken into account. But a more complicated splicing pattern of mRNA could link, in one open reading frame, areas with small coding capacity scattered all along the genome. If region 3 which spans in the single-stranded part of the genome is actually translated into protein, this can happen only after DNA synthesis of the missing strand.

In regions 5, 6, 7 and 8, the situation appears to be very different. The number of codons located downstream of the first ATG are respectively 154, 832, 389 and 212. Region 6 (2,553 nucleotides long) contains 9 ATG codons. The first and second are located at 57 and 1,296 nucleotides respectively from the beginning of this region. The seven other ATGs are regularly distributed in the second half of this region. In the case where translation is initiated at the first ATG, a protein of MW 90,000 might be synthesised. DNA polymerases and reverse transcriptases have a MW of that order<sup>22,33</sup>, so it is tempting to suggest that the DNA polymerase found within the virion could be coded by this region.

By correlating the amino acid sequences of both ends of the HBs Ag polypeptide I<sup>24</sup> and the nucleotide sequence of the *Eco* HBV DNA, we are able to localise the gene coding for this polypeptide in region 7. This gene, called *S* gene, was previously located between positions, 95.1 and 73.6 on the genome map<sup>11</sup>. The ATG (position 3,030) corresponding to the N-terminal extremity of the polypeptide I is the third of region 7. It is located 618 nucleotides from the beginning of this region, whereas the two other ATGs (positions 337 and 13) are at 129 and 453 nucleotides from the beginning. The fact that the ATG 3030 is preceded by two other ATGs in the same open reading frame deserves some additional comment. Three explanations can be given: (1) Region 7 is completely translated and gives rise to a long polypeptide different from polypeptide I but whose C-terminal amino acid sequence is identical to polypeptide I. A similar situation occurs with protein VP2 and VP3 of SV40<sup>25,26</sup> and the 33,000 and 100,000 MW proteins of adenovirus<sup>27</sup>. (2) Region 7 is completely translated into a precursor protein of polypeptide I. (3) The region upstream of the third ATG is not translated. At the present time, one cannot choose between the

**Fig. 3** (Below and opposite) Nucleotide sequence of *Eco* HBV DNA. Stop codons in the various reading frames are indicated in the following manner: ○ stop codon in phase 1; ● in phase 2; + in phase 3. The position of *HincII*, *Bam*HI, *Bgl*II and *Xho*I restriction sites are indicated.

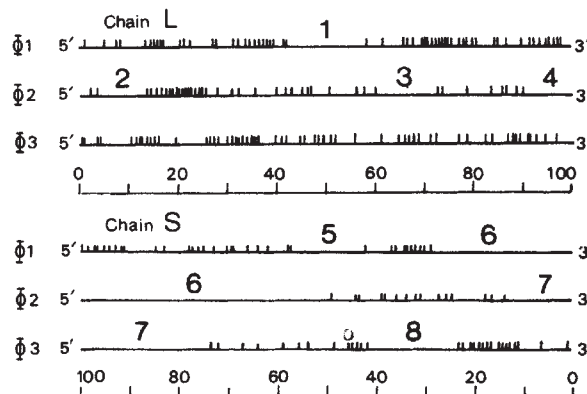


three hypotheses. Nevertheless, the first hypothesis could partially explain the presence of large polypeptides found in small amount in the HBs Ag preparations<sup>24</sup>.

Translation of the terminal nucleotide sequence of region 8 would give rise to a polypeptide having at its C-terminal end a repeated sequence of 9 amino acids: Ser-Pro-Arg-Arg-Arg-Arg-Ser-Glu-Ser. The last amino acid of the first sequence, Ser, would be the first one of the second sequence. In the corresponding DNA sequence, however, the only repetition is TCTCAA (Ser-Glu). Beyond the ATG codon, region 8 contains 24 arginine codons—11% of the total codons. In the last 35 codons there are 16 arginine codons. This large proportion of basic amino acids is similar to that observed in histone, which suggests that the translational product of region 8 could be a polypeptide with a high affinity for HBV DNA. Therefore, if this region is translated, one could envisage that the HBc Ag could be coded by this DNA sequence.

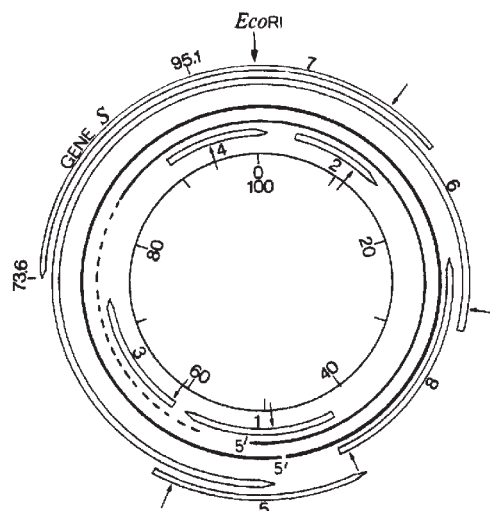
## Search for signal sequences

From a comparative analysis of the primary structure of viral or cellular mRNAs and of chromosomal DNA fragments, some remarkable DNA sequences were found. In several circumstances, a sequence identical or similar to 5'TATAAA was observed in front of the transcribed region (ref. 28 and D. Hogness and M. Goldberg, personal communication), while the sequence AAUAAA has been found at the 3' end of mRNA, near the poly(A)tail<sup>29-31</sup>. On the other hand, it has been shown that during the initiation of translation the 3'OH end of 16S ribosomal RNA hybridises to the 5' end of prokaryotic mRNA through the CCUCC sequence<sup>32</sup>. The occurrence of a similar phenomenon has not been demonstrated in eukaryotic cells. Nevertheless, sequences able to hybridise to the 3'GCGGAAGGA sequence of the 18S ribosomal RNA have been observed in some eukaryotic mRNAs<sup>28,33-35</sup>. Therefore, we looked for these sequences in the *Eco* HBV DNA. The 5'TATAAA sequence was found only at position 1,269 on the S strand at 335 nucleotides before region 6. It could therefore correspond to a signal relating to the initiation of transcription of this very large open region. In that case, since this sequence is followed by three ATGs (1,089, 1,022, 998) all in a closed reading frame, a splicing of the corresponding mRNA should



**Fig. 4** Diagram showing the localisation of the nonsense codons on chains L and S of the *EcoHBV* DNA. Three reading frames, called  $\Phi 1$ ,  $\Phi 2$  and  $\Phi 3$ , were defined from the 5' end of each DNA strand. On the L chain,  $\Phi 1$  is defined by its first triplet AAT,  $\Phi 2$  by ATT and  $\Phi 3$  by TTC. On the S chain,  $\Phi 1$  is defined by its first triplet CCA,  $\Phi 2$  by CAC and  $\Phi 3$  by ACA. The viral DNA is circular and its length in nucleotides (3,182) is not a multiple of 3. Therefore, going through the *EcoRI* site phase  $\Phi 1$  is change to phase  $\Phi 2$ , phase  $\Phi 2$  to phase  $\Phi 3$  and phase  $\Phi 3$  to phase  $\Phi 1$ . Vertical bars represent stop codons. Numbers 1 to 8 define the areas with an open reading frame arbitrarily chosen with a length above 100 codons. Region 1 goes from nucleotide 1,351 to 1,860, region 2 from nucleotide 119 to 445, region 3 from 1,904 to 2,350, region 4 from 2,855 to 17, region 5 from 1,847 to 1,350, region 6 from 935 to 1 and from 3182 to 1565, region 7 from 466 to 1 and from 3,182 to 2353, region 8 from 1,392 to 736.





**Fig. 5** Localisation of the open reading frames on the viral genome. The structure of the genome is shown as previously described<sup>8</sup>. The *EcoRI* site is the origin of the physical map. Block arrows define the open reading frames on the two viral DNA strands. The small arrows indicate the ATG codon located closest to the beginning of the open region. The *S* gene is located between positions 95.1 and 73.6 and is included in region 7.

take place to link its 5' end to a nucleotide sequence corresponding to the open reading frame of region 6. Sequences which differ by only one nucleotide were found 36 times on the *S* strand and 45 times on the *L* strand. The 5'AATAAA sequence occurs once on the *L* strand at position 689, 243 nucleotides after the stop codon of region 2. This sequence is not found in chain *S* even at the end of gene *S* which is known to code for polypeptide 1. Two sequences, 5'GCCTTCCT and CGCCTTCC, able to hybridise with the 3' end of 18S RNA ribosomal, were found at positions 61 and 417 on the *L* strand. Sequences able to hybridise with 6 or 7 nucleotides of the 3'OH end of this RNA were found once on the *L* chain and 8 times on the *S* chain. Among these, the CCTTCC sequence located at position 3,176 on the *S* strand follows a short palindromic sequence 5'GTGGAATTCCAC 3' starting at position 8. This

feature could be related to the expression of the *S* gene located downstream.

Containment conditions for this study were recommended by the French National Control Committee. Growth of recombinant bacteriophages was done in L3B1 conditions. We thank Dr A. Fritsch for helpful discussion and reviewing the manuscript, Dr R. Studen for computer work, Dr A. Maxam for sending us the revised version of the Maxam and Gilbert sequencing method before publication, and Mrs Gobet for technical assistance. This work was supported by INSERM and CNRS.

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## Design, synthesis and characterisation of a 34-residue polypeptide that interacts with nucleic acids

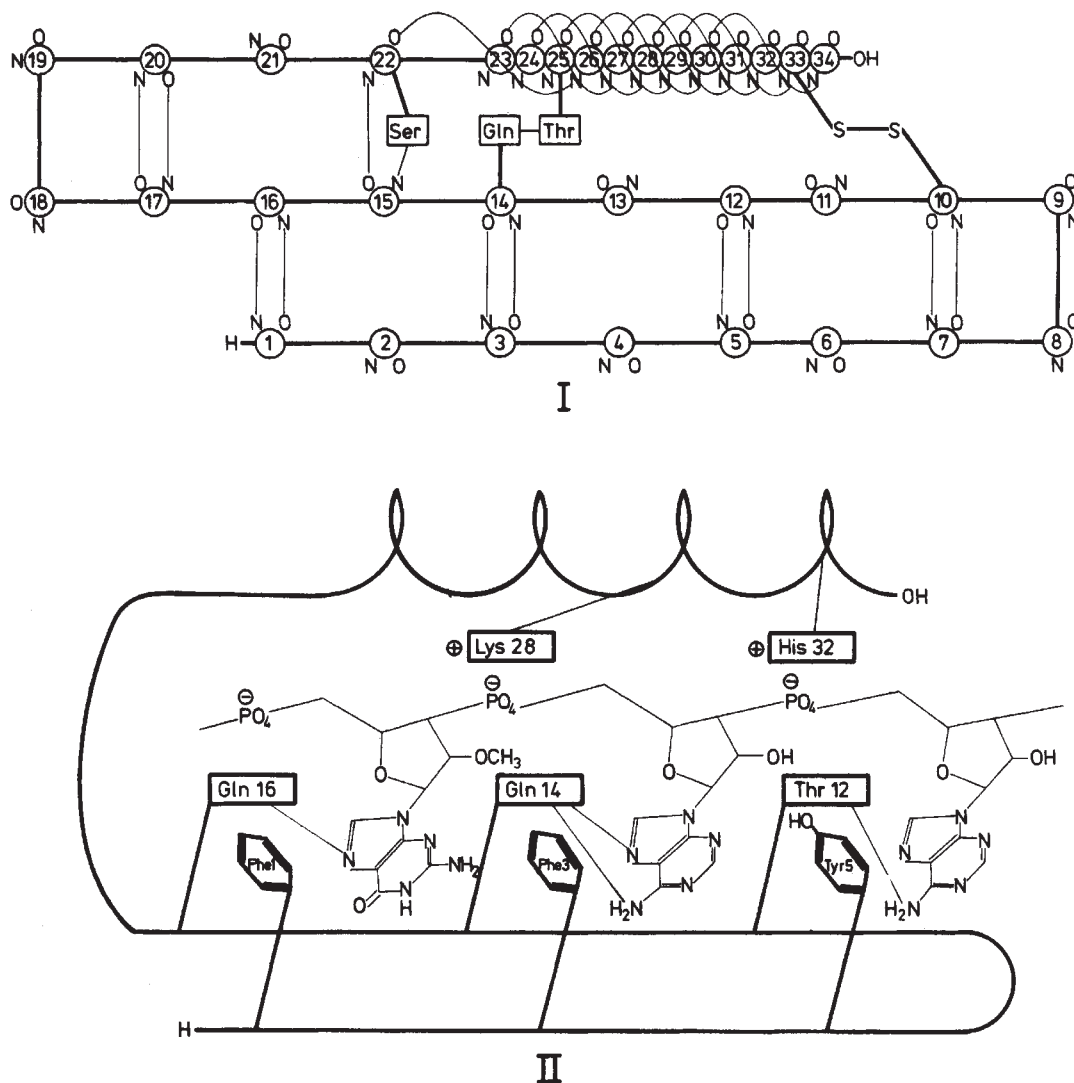
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*Based on secondary structure prediction rules and model building a neutral artificial 34-residue polypeptide with potential nucleic acid-binding activity was synthesised. This peptide and its covalent dimer showed strong interaction with cytidine phosphates and single-stranded DNA. The dimer had considerable ribonuclease activity with high preference for cleavage at the 3'-end of C.*

**BASED** on the known X-ray structures of proteins, rules were proposed by various authors (see ref. 1 for review) to describe the relationship between primary and secondary structure of a protein molecule. These rules may be applied to predict the secondary structure of proteins of which only the amino acid sequence is known. An example of this is the suggested succession of the secondary structural elements ( $\alpha$ -helix,  $\beta$ -strand, reverse turn) in *Escherichia coli lac* repressor<sup>2,3</sup>. Proof or disproof of the proposed secondary structure will come from X-ray diffraction studies on crystalline *lac* repressor.

**Fig. 1** Proposed secondary structure of synthetic 34-residue polypeptide (I) and possible interactions with the trinucleotide  $m^2$  GAA (II). I, Thin lines represent hydrogen bonds between the  $\beta$ -strands and within the  $\alpha$ -helix, amino acid symbols in boxes are side chains involved in intramolecular hydrogen bonds. In the model, the helix axis lies slightly above the hydrophobic side of the  $\beta$ -sheet. The primary structure of the 34-residue polypeptide is given in the text. II, Heavy lines indicate the course of the peptide chain, thin lines show the trinucleotide. The side chains of the amino acid residues in boxes are proposed to be the main contributors to the interactions between the synthetic polypeptide and the trinucleotide. The ribose-phosphate backbone runs above the two parallels formed by the 10-17 strand and the helix axis. The bases point downwards onto the two-strand sheet formed by residues 1-7 and 10-17.



The secondary structure prediction rules may also be used to design functional polypeptides that do not occur naturally but that may be useful for scientific and industrial purposes. Although these rules will certainly become refined and more reliable as more X-ray structures of proteins become known, we decided to design a nucleic acid-binding polypeptide based on the present prediction methods. This system was chosen because of the great importance of nucleic acid-protein interactions in biology<sup>4</sup>.

### Rationale of the amino acid sequence chosen

The structure and the amino acid sequence of the polypeptide that could have the desired properties were determined in part by model building. The nucleotide sequence of the anticodon of yeast tRNA<sup>Phe</sup>,  $m^2$  GAA (refs 5-7), was used as the ligand around which the backbone of a peptide chain was to be folded in such a way as to produce a shallow depression as binding site. This simple structure seemed to be formed best by a 34-residue polypeptide containing a  $\beta$ -strand, a reverse turn, an antiparallel  $\beta$ -strand, a second reverse turn, and an  $\alpha$ -helix running antiparallel to the second  $\beta$ -strand as viewed from the  $\text{NH}_2$ -terminus to the  $\text{COOH}$ -terminus. Although this  $\beta\beta\alpha$  arrangement was similar to the  $\beta\alpha\beta$  units that make up the  $\text{NAD}^+$ -binding fold of the dehydrogenases<sup>8,9</sup>, the proposed mode of interaction between the synthetic polypeptide and the trinucleotide was quite different.

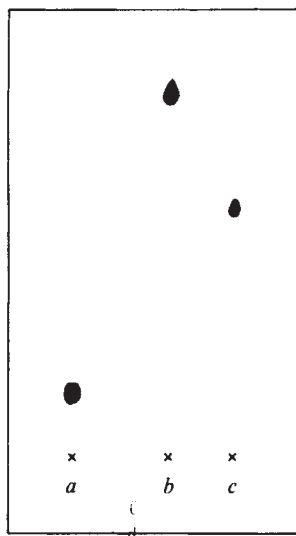
The next step was choosing an amino acid sequence that, based on the prediction method of Chou and Fasman<sup>10</sup>, had the

potential to give the proposed folding and to form a sufficient number of specific noncovalent bonds with the ligand. Two additional prerequisites had to be fulfilled. First, the binding site was to be predominantly hydrophobic containing a few residues the side chains of which could act as hydrogen bond donors or acceptors; and second, binding was to be achieved mainly through specific hydrogen bonds and van der Waals contacts and only to a smaller extent through electrostatic interactions. The sequence chosen was as follows:

1 Phe-Thr-Phe-Thr-Tyr-Thr-Asp-Pro-Asn-Cys-Gln-Thr-Gly-  
15 Gln-Gly-Gln-Asn-Pro-Asn-Gly-Ile-Ser-Glu-Pro-Thr-Ala-  
30 Ala-Lys-Val-Ala-Gln-Ala-His-Cys-Ala  
34

The proposed secondary structure of this peptide and its possible interactions with the ligand are shown in Fig. 1. Residues Phe 1 to Asp 7 were assumed to have an extended structure with the side chains of Phe 1, Phe 3, and Tyr 5 forming part of the binding site and allowing stacking interactions or intercalation with the bases of the trinucleotide ligand and the side chains of Thr 2, Thr 4, and Thr 6 pointing in the opposite direction, thus allowing interactions with the solvent. Residues 7-10 could form a  $\beta$ -turn and residues 10 to 17 could form the second strand of a  $\beta$ -sheet with the side chains of Thr 12, Gln 14, and Gln 16 hydrogen-bonded to the base moieties of the trinucleotide in a hydrophobic environment. Following the second





**Fig. 2** Electrophoresis (a) and TLC (b, c) of purified synthetic 34-residue polypeptide. Electrophoresis was in 0.1 M pyridine-acetate, pH 5.8, on a precoated cellulose thin layer plate (20 × 20 cm) at 600 V for 50 min. The mobility of the spot was 2.3 cm. The product was also homogeneous on electrophoresis at pH 3.4. TLC was on precoated silica gel plates in ethyl acetate/acetic acid/pyridine/water (3:1:5:3) (b,  $R_F$  0.80) and in *n*-butanol/acetic acid/pyridine/water (10:3:10:12) (c,  $R_F$  0.50). Spots were detected by the Pauly spray.

$\beta$ -turn, possibly produced by residues 17–20, a short third strand and the COOH-terminal  $\alpha$ -helix comprising residues 23–34 completed the proposed secondary structure of the synthetic 34-residue polypeptide. The side chains of Ile 21, Ala 26, and Val 29 could be part of the hydrophobic binding site and the side chains of Lys 28 and His 32 could form salt bridges with the phosphate moieties of the ligand although the contribution of the latter interactions to the stability of the peptide-trinucleotide complex was probably weak, as in the model these salt bridges were on the surface of the complex. The disulphide bond between half-cystines 10 and 33 seemed to stabilise the proposed structure of the 34-residue polypeptide and its inter-

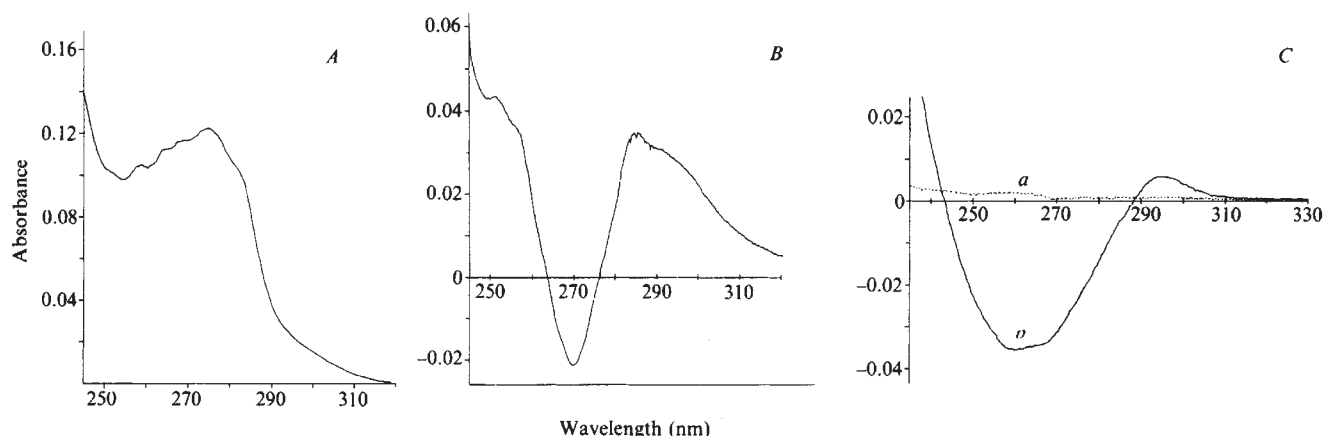
actions with the trinucleotide. It also brought the side chains of three potential active site residues (Asp 7, Thr 12, and His 32) into spatial proximity.

The model of the 34-residue polypeptide-trinucleotide complex suggested the possibility of aggregation, either through continuation of the stacking interactions to the left of the NH<sub>2</sub>-terminus of the peptide (Fig. 1, II) or through an antiparallel face-to-face arrangement of the NH<sub>2</sub>-terminal heptapeptide portions of two 34-residue polypeptide molecules mediated by intermolecular hydrogen bonds between residues 2, 4 and 6 of one molecule (Fig. 1, I) and residues 6, 4 and 2, respectively, of the other.

## Solid phase synthesis and purification of the 34-residue polypeptide

The 34-residue polypeptide was synthesised by the solid phase method<sup>11,12</sup> as described previously<sup>13</sup>. The amino acid composition of an acid hydrolysate of the protected peptide resin is given in Table 1. The 34-residue peptide was deprotected and cleaved from the resin by anhydrous HF in the presence of excess anisole at 0 °C and was extracted by 1 M acetic acid. After lyophilisation it was dissolved in 0.05 M NH<sub>4</sub>HCO<sub>3</sub> adjusted to pH 8.8 with concentrated NH<sub>4</sub>OH. The solution was stirred several hours and then desalted on a Bio-Gel P-2 column. The yield of crude 34-residue peptide was 67% based on the amount of COOH-terminal alanine esterified to the resin at the beginning of the synthesis.

The synthetic product was reduced with mercaptoethanol, reoxidised in dilute solution at pH 8.55 (ref. 13), and fractionated on a Sephadex G-50 column (78.5 × 2.7 cm) in 0.05 M NH<sub>4</sub>HCO<sub>3</sub>. The major fractions obtained were a polymer (yield, 5% of the amount applied to the column), a tetramer (yield, 19%), and the monomer (yield, 41%) which, after rechromatography, eluted between 136 and 154 ml, 211 and 247 ml (corresponding to the elution volume of lysozyme), and 272 and 308 ml, respectively. When 1 M HCOOH was used as eluant, the elution volume of the tetramer was increased to 240 to 270 ml but that of the monomer remained unchanged. This indicated that the tetramer was formed through association of two dimers the monomeric subunits of which were linked covalently by two intermolecular disulphide bonds. Whether the S-S bridges extended between Cys 10 and Cys 33 of one subunit and Cys 10 and Cys 33 or Cys 33 and Cys 10, respectively, of the



**Fig. 3** A, B, UV difference spectrum of synthetic 68-residue polypeptide in the presence of cytidine 2':3'-cyclic phosphate. Measurements were performed in a Cary 118 spectrophotometer. A, Spectrum of the 68-residue polypeptide (0.02 mM solution in 0.1 M sodium acetate, pH 5.3;  $d$ , 1 cm). B, Spectrum of the mixture after addition of the nucleotide to a concentration of 0.1 mM in both the control and the sample. C, UV difference spectrum of synthetic 34-residue polypeptide in the presence of single-stranded DNA. Single-stranded DNA was prepared from double-stranded calf thymus DNA by alkali denaturation. The 34-residue polypeptide was dissolved in buffer, pH 6.0, containing 0.06 M sodium chloride, 0.04 M sodium cacodylate, and 0.001 M EDTA. The separate compartments of two tandem cuvettes ( $d$ , 0.877 cm) were filled with 0.75 ml DNA solution (0.034 mg ml<sup>-1</sup>) and 0.75 ml of peptide solution (0.24 mg ml<sup>-1</sup>), respectively. After recording the base line (a), the contents of the two compartments of one of the cuvettes were mixed and the spectrum of the mixture was measured against that of the separate components (b). The UV difference spectrum produced by a mixture of the 68-residue material and single-stranded DNA was almost identical to that shown here.

other subunit or whether a mixture of both disulphide isomers was present, has not yet been determined.

Further purification of the 34-residue polypeptide and its dimer was achieved best by adsorption chromatography of 10-mg samples on a silica gel column (50 × 2.2 cm) in *n*-butanol/acetic acid/pyridine/water (20:6:20:24). The monomeric material that had the required amino acid composition (Table 1) was eluted between 102 and 109 ml in 35% yield based on the amount applied to the column. The major impurity eluting between 114 and 122 ml was a deletion peptide in which one threonine and one phenylalanine residue were missing. The purified monomer was homogeneous electrophoretically and chromatographically (Fig. 2). Fractionation of the dimer on silica gel gave similar results.

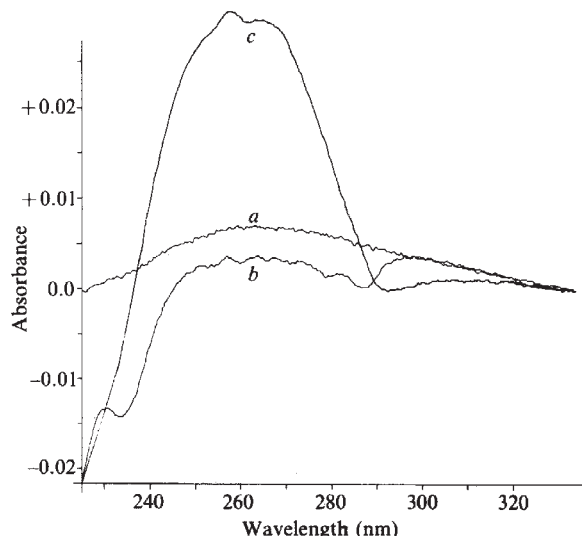
## Interaction of the synthetic polypeptides with cytidine phosphates and single-stranded DNA

To visualise possible interactions between the purified synthetic 68- and 34-residue polypeptides and the 2':3'-cyclic phosphates of adenosine, cytidine, guanosine, and uridine, UV spectra were recorded in a Cary 118 double-beam spectrophotometer. First, the 68-residue polypeptide was measured against the solvent, 0.1 M sodium acetate, pH 5.3. Then the nucleotide was added to both cuvettes in a small volume of buffer and the spectrum was recorded again between 320 and 250 nm. There was almost no change of the spectrum of the peptide in the presence of adenosine, guanosine, or uridine phosphate. In the presence of cytidine 2':3'-cyclic phosphate, however, the spectral change was drastic (Fig. 3, A, B). The absorption maximum of the peptide was shifted from 274.5 to 285 nm and was quenched considerably. The spectrum of the mixture also showed a large negative peak approximately at the position of the absorption maximum of the nucleotide alone (268 nm). Addition of cytidine 2':3'-cyclic phosphate to the 34-residue peptide and to its [Ala<sup>10</sup>, Ala<sup>33</sup>]-analogue which cannot form disulphide bonds, produced only a small decrease of the optical absorption of the two peptides.

The strong interaction between the 68-residue polypeptide and cytidine phosphate indicated by the UV difference spectrum (Fig. 3, A, B) was confirmed through determination of the dissociation constant of the polypeptide-nucleotide complex. Using the method of Hummel and Dreyer<sup>14</sup> and varying the concentration of 2'-CMP from 0.02 mM to 0.15 mM (in the presence of 0.1 mM 2'-CMP, for instance, the concentration of the polypeptide-ligand complex was  $9.9 \times 10^{-6}$  M and that of free polypeptide  $5 \times 10^{-7}$  M), the  $K_D$  was found to be  $5 \times 10^{-6}$  M in 0.01 M sodium acetate, pH 5.3 and was of the same order of magnitude as the  $K_D$  of the RNase A-2'-CMP complex<sup>15</sup>. By this method the 68-residue peptide bound 2'-CMP 30 times more strongly than did the 34-residue peptide.

The binding properties of the synthetic peptides were investigated further by affinity chromatography. Poly(A)-Sephrose 4B did not retard the elution of the 34-residue polypeptide but retarded considerably part of the 68-residue polypeptide applied—75% of the dimer was eluted at pH 5.3 at a concentration of 0.03 M to 0.07 M sodium acetate, the remaining 25% was eluted at 0.1 M to 0.24 M sodium acetate, pH 5.3. No differences in amino acid composition could be detected, so the two fractions may represent conformational isomers, possibly the disulphide isomers, of the synthetic 68-residue polypeptide. Poly(U)-Sephrose 4B and Blue Sepharose CL-6B, which has some affinity for proteins<sup>16</sup> possessing the NAD<sup>+</sup>-binding fold<sup>8,9</sup>, did not bind either of the two synthetic peptides.

Difference spectral measurements showed that the absorption of the mixtures of 68- and 34-residue polypeptide with double-stranded calf thymus DNA deviated only slightly from the sum of the absorptions of the separate components. The UV difference spectra obtained in the presence of single-stranded DNA, however, indicated strong polypeptide-nucleic acid interactions (Fig. 3, C). The main features of these spectra were



**Fig. 4** Spectral evidence for the digestion of RNA by the synthetic 68-residue polypeptide. tRNA<sup>Phe</sup> and 68-residue peptide were dissolved in buffer, pH 6.0, containing 0.1 M NaClO<sub>4</sub>, 0.005 M sodium cacodylate, and 0.0001 M EDTA. The separate compartments of two tandem cuvettes (*d*, 0.877 cm) were filled with 0.75 ml of tRNA<sup>Phe</sup> solution (0.35 μg ml<sup>-1</sup>) and 0.75 ml of peptide solution (0.04 mg ml<sup>-1</sup>), respectively. After recording the base line (*a*), the contents of the two compartments of one of the cuvettes were combined and the spectrum of the mixture was measured against that of the separate components immediately after mixing (*b*) and again 8 h later (*c*). The reaction took place at 22 °C.

a new peak with the absorption maximum at 295 nm and a depression with the absorption minimum extending from 258 to 267 nm.

## Nuclease activity of synthetic 68- and 34-residue polypeptides

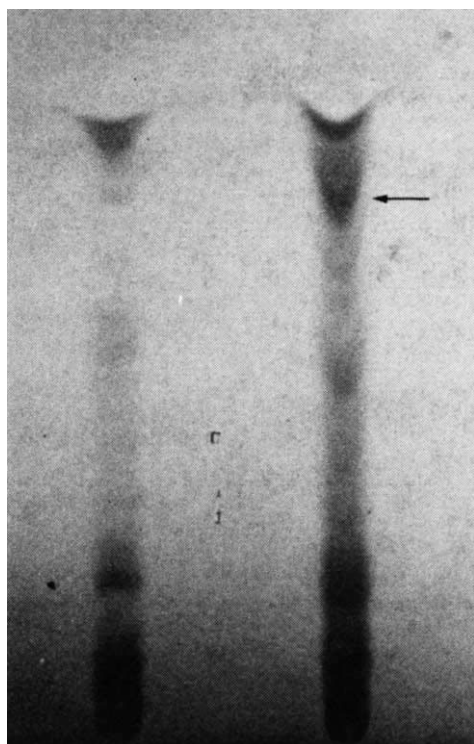
To test whether the synthetic peptides interacted with the ligand around which the model had been built, UV difference spectra of the mixtures of 68- and 34-residue polypeptide with tRNA<sup>Phe</sup> in which the anticodon sequence m<sup>2</sup>GAA is in an exposed position were recorded.

Figure 4 shows that the spectral change observed immediately after combining solutions of 68-residue polypeptide and tRNA<sup>Phe</sup> was small (curve *b*) indicating weak interaction between the two components of the mixture. However, when the spectrum was recorded again 8 h later a new broad peak with the maximum absorption at approximately 260 nm (curve *c*) was found. This type of spectral change is seen when RNA is digested by ribonucleases.

That the increase of the optical absorption around 260 nm was indeed due to a ribonuclease activity of the 68-residue polypeptide was confirmed by TLC of an aliquot of the contents of the cuvette. The digestion pattern obtained was similar to that shown in Fig. 5. In the Anfinson assay<sup>17,18</sup> using tRNA from baker's yeast as substrate the specific activity of the synthetic 68-residue polypeptide was 2.5% that of natural RNase A.

Comparison of the patterns produced on digestion of tRNA by RNase A and the synthetic enzyme (Fig. 5, left and right, respectively) showed differences mainly between the products of high *R<sub>F</sub>* or short chain length. This indicated that either the rates of the cleavage reactions or the substrate specificities of the two nucleases were different. Using poly(A), poly(C), poly(G) and poly(U) as substrates, differences in the cleavage rates and in the substrate specificity were indeed noticed in a semiquantitative assay. The 68-residue polypeptide digested the four polynucleotides with decreasing rates in the following order,





**Fig. 5** TLC separation of the digestion products of tRNA produced by the action of synthetic 68-residue polypeptide (right) and natural RNase A (left). tRNA (0.20 mg each) was incubated with RNase A (0.1  $\mu$ g) and 68-residue polypeptide (0.03 mg), respectively, in 30  $\mu$ l of buffer, pH 6.0, at 22 °C. Aliquots (3  $\mu$ l) of the reaction mixtures were chromatographed every 12 h until the digestion patterns remained unchanged (the patterns shown were obtained after a 93.5-h reaction time). Chromatograms were developed on silica gel F<sub>254</sub>-coated thin layer plates in isopropyl alcohol/ammonium hydroxide/water (14:1:5). Spots were detected under UV light. The arrow indicates the position of free cytidine 2':3'-cyclic phosphate. A control of undigested tRNA (not shown) gave a single spot at the origin. The buffer for the incubations contained 0.1 M NaClO<sub>4</sub>, 0.005 M sodium cacodylate, and 0.0001 M EDTA, adjusted to pH 6.0.

poly(C)  $\gg$  poly(A)  $>$  poly(U)  $\gg$  poly(G). Using RNase A as enzyme, this order became poly(C)  $>$  poly(U)  $\gg$  poly(A); poly(G) was not cleaved at all. The fact that cytidine phosphates were bound tighter by the 68-residue polypeptide than other nucleotides was reflected by the relatively fast depolymerisation of poly(C) in the presence of the synthetic enzyme. The nucleoside 2':3'-cyclic phosphates seemed to be the final digestion products. The maximum of the rate of depolymerisation was between pH 6 and pH 8. But even in 0.1 M formic acid the 68-residue polypeptide had considerable nuclease activity; in identical conditions 0.1 M formic acid alone did not hydrolyse poly(C).

The activity of the two fractions obtained by affinity chromatography of the 68-residue polypeptide on poly(A)-Sephrose 4B was the same within the limit of error of the assay method using tRNA as substrate. Whether the tighter-binding fraction that eluted at sodium acetate concentrations of 0.1 to 0.24 M had increased nuclease activity towards poly(A) has not been tested.

The 34-residue polypeptide possessing one intramolecular disulphide bond was less than 4% as active as the 68-residue polypeptide. This was probably because of weaker binding of nucleic acids by the monomer. As controls, insulin and bovine serum albumin were completely inactive in these assays.

In assays for proteolytic (tryptic, chymotryptic, peptic) and glycolytic (lysozyme) activity both the 68- and the 34-residue polypeptide were inactive.

The possibility that the low ribonuclease activity of the 34-residue polypeptide might be due to incomplete separation from the more active 68-residue peptide was excluded by preliminary results obtained with an [Ala<sup>10</sup>, Ala<sup>33</sup>]-analogue of the 34-residue polypeptide. The nuclease activity of this analogue suggested that the disulphide bond was not essential for the structure and biological function of the 34-residue polypeptide and that the amino acid sequence chosen from model building could indeed fold in such a way as to allow interactions with nucleotides and nucleic acids. Dimerisation of the Cys-containing monomer through formation of two intermolecular disulphide bonds, however, seemed to produce a cleft or pocket in which the essential groups were in a more favourable position for binding and catalysis.

Chemical modification has been used successfully to determine active site residues and to propose catalytic pathways of ribonucleases<sup>19</sup>. Thus, iodoacetic acid was found to carboxymethylate at pH 5.5 the side chains of His 119 and His 12 of RNase A<sup>20</sup> and of Glu 58 of RNase T<sub>1</sub><sup>21</sup> to give inactive enzymes. When the synthetic 68-residue polypeptide was submitted to similar conditions<sup>20</sup> for 12 h, no carboxymethylation of the positively charged amino acid residues (Lys 28, His 32) took place as determined by electrophoresis and by amino acid analysis of an acid hydrolysate. The electrophoretic mobility of the 68-residue polypeptide, incubated with iodoacetic acid, on cellulose-coated thin layer plates in 1.2 M formic acid, 2 M urea, pH 2.2, was unaltered and no N<sup>1</sup>- or N<sup>3</sup>-carboxymethyl-histidine was detected by amino acid analysis. Carboxymethylation of the 68-residue polypeptide at pH 8 gave identical results. However, alkylation of the side chain of Asp 7 or Glu 23 would have gone undetected by these methods. Therefore, the reaction was repeated using iodo[2-<sup>14</sup>C]acetic acid. After 17 h at pH 5.75 only 1 out of 50 protein molecules seemed to be labelled. This was consistent with the result of the nuclease assay in which there was no noticeable loss of activity of the 68-residue polypeptide after iodoacetic acid treatment. Thus the geometry of the active site of the synthetic enzyme is apparently different from that of the active sites of RNase A and RNase T<sub>1</sub>.

## CD spectra of the synthetic polypeptides

To establish whether the synthetic peptides adopted the hypothetical secondary structure in aqueous solution spectral analyses were performed. The predicted structure (Fig. 1, I) suggested pronounced Cotton effects between 185 and 230 nm. In addition, the presence of phenylalanine and tyrosine residues in the NH<sub>2</sub>-terminal region of the peptides could give rise to

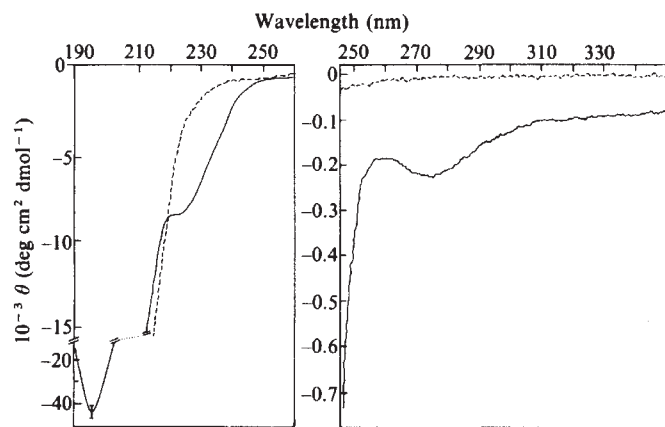
**Table 1** Amino acid composition of synthetic 34-residue polypeptide

|     | Expected | Protected peptide resin | Purified peptide |
|-----|----------|-------------------------|------------------|
| Asp | 4        | 4.5                     | 4.2              |
| Thr | 5        | 3.5*                    | 5.1              |
| Ser | 1        | 0.5*                    | 1.0              |
| Glu | 5        | 5.0                     | 4.9              |
| Pro | 3        | 2.8                     | 3.1              |
| Gly | 3        | 3.0                     | 3.0              |
| Ala | 4        | 4.6                     | 4.1              |
| Cys | 2        | —†                      | 2.0‡             |
| Val | 1        | 1.0                     | 1.0              |
| Ile | 1        | 0.9                     | 1.0              |
| Tyr | 1        | 0.8                     | 0.8              |
| Phe | 2        | 1.8                     | 1.9              |
| Lys | 1        | 1.1                     | 1.0              |
| His | 1        | 1.0                     | 1.0              |

\* Peptide resin hydrolyses usually give low values for Thr and Ser.

† Not determined.

‡ After performic acid oxidation and acid hydrolysis.



**Fig. 6** CD spectrum of the 34-residue peptide. Measurements in the near and far UV region were carried out in a Dichrographe II with high sensitivity accessory and thermostated cuvette compartment using 0.1 mm to 10 mm cells. To determine molar concentrations absorption spectra were monitored in a Zeiss DMR 10 spectrophotometer. The peptide was dissolved in 0.01 M sodium citrate, pH 5.3 (solid line), and in 0.001 M sodium citrate–6 M guanidinium hydrochloride (dashed line), respectively.

dichroic absorption in the range of aromatic absorption at  $\sim 280$  nm.

The 68-, 34-, and [Ala<sup>10</sup>, Ala<sup>33</sup>]-34-residue polypeptides showed similar optical properties in the aromatic region. In the far UV the presence or absence of cystine residues generated quantitative differences. A helical contribution was represented by a minimum at  $\sim 222$  nm. Other characteristic features of protein CD spectra seemed to be shifted towards the UV region giving rise to a second minimum at 197 nm. The corresponding maximum was below 185 nm. A representative example is given in Fig. 6 (solid line) illustrating the overall CD profile of the 34-residue polypeptide. On addition of 6 M guanidinium hydrochloride (Fig. 6, dashed line) the CD bands in the aromatic absorption range and in the far UV region of the spectrum disappeared indicating a marked decrease in original secondary structure. Quantitative evaluation of helix,  $\beta$ -structure, and  $\beta$ -turn contents was not possible because of the specific interactions within the relatively small polypeptide molecule. Experiments dealing with solvent effects and ligand binding will be published elsewhere<sup>22</sup>.

## Discussion

Two problems must be solved before it becomes possible to design artificial proteins with novel properties for laboratory, medical and industrial use. First, assembly of the secondary structural elements to give the biologically active tertiary structure is at present only poorly understood; and second, chemical synthesis of proteins is still extremely difficult if not impossible. However, it is reasonable to assume that these obstacles will be overcome. Artificial proteins could also be produced by a genetic approach through expression of synthetic genes in bacteria<sup>23</sup>. An alternative route to artificial proteins is the thermal copolymerisation of amino acids yielding a mixture of proteinoids of varying composition and size. Such polymers have non-random amino acid composition<sup>24</sup> and are capable of catalysing chemical reactions such as hydrolysis, decarboxylation or amination<sup>25</sup>. Although the catalytic activities are weak, some thermal polyanhydro- $\alpha$ -amino acids do show substrate specificity and stereoselective action<sup>25</sup>. Isolation from the proteinoid mixture of single species possessing interesting enzymatic properties may be a formidable task and has not yet been reported.

Reproducible preparation of artificial proteins with distinct properties can probably best be achieved by a planned synthesis. An example for the latter approach is the synthesis by Chakravarty *et al.*<sup>26</sup> of an active lysozyme model consisting of only 10 amino acid residues. Based on the work of Kotelchuck

and Scheraga<sup>27</sup>, Chakravarty *et al.* designed a decapeptide that, aided by the polymeric nature of the substrates used, could adopt  $\alpha$ -helical conformation displaying the essential unionised and ionised carboxyl groups in a suitable environment. This decapeptide showed weak glycosidase activity.

In the nucleic acid-binding polypeptide described here it is likely that stacking of the aromatic side chains with the bases of the nucleotides and hydrogen bonds in a hydrophobic environment contributed to the interaction energy. As the synthetic peptide was neutral, it possibly resembled in its properties several of the non-histone chromosomal proteins. The fact that cytidine phosphates were bound with relatively high specificity whereas interaction with the trinucleotide sequence around which the model has been built was weak, may have been due to differences between the proposed and the actual folding of the synthetic polypeptide. Preliminary CD measurements showed distinct CD bands in the aromatic and in the far UV absorption range that were extinguished in the presence of 6 M guanidinium hydrochloride. To determine secondary and tertiary structure more precisely we shall attempt crystallisation of the 34-residue polypeptide.

The synthetic nucleic acid-binding polypeptide also had ribonuclease activity with high preference for the cleavage of 3',5'-phosphodiester bonds following 3'-linked cytidine residues. Dimerisation of the 34-residue polypeptide through formation of two intermolecular disulphide bonds improved the binding of cytidylic acid and enhanced the ribonuclease activity. This activity was not caused by microbial contamination since proteins such as insulin and bovine serum albumin were completely inactive in the nuclease assays after they had been passed through the same columns that were used for the purification of the synthetic polypeptides. As nucleases like RNase T<sub>1</sub>, RNase A, or staphylococcal nuclease are among the shortest enzymes known, their action may be more easy to simulate by designed synthetic models than that of larger and more complex enzymes. Furthermore, the presence of a histidine residue in a position favourable for catalysis may have been essential for the nuclease activity of the 34-residue polypeptide because histidine residues are frequently found in the active site of hydrolytic enzymes.

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# letters

## X-ray and [Fe XIV] 5,303 Å emission from Puppis A supernova remnant

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**Recently, Lucke *et al.*<sup>1</sup> found [Fe XIV] 5,303 Å emission from Puppis A to be much less intense than that expected from the X-ray data given by Zarnecki *et al.*<sup>2</sup>. We propose here that this discrepancy can be removed by taking into account the nonequilibrium state of ionisation within the X-ray source.**

Interpreted under the assumption of ionisation equilibrium, the X-ray spectrum of Pup A consists of two components which have temperatures of 13 and  $2.5 \times 10^6$  K (ref. 2). Therefore, if ionisation equilibrium indeed holds, the low-temperature plasma should emit [Fe XIV] 5,303 Å photons as observed in the solar corona. However, the measured [Fe XIV] emission<sup>1</sup> has been too weak to be in harmony with the volume emission measure<sup>2</sup> of the low-temperature plasma. Lucke *et al.*<sup>1</sup> have argued that the most plausible way of reducing this discrepancy to within experimental error is probably by assuming the maximum temperature of  $3 \times 10^6$  K allowed by the X-ray data<sup>2</sup>. This interpretation, however, does not seem to be tenable, since ionisation equilibrium is unlikely to hold within the X-ray source. In fact, from a calculation<sup>3</sup> of time-dependent ionisation of oxygen for a post-shock temperature of  $2.8 \times 10^6$  K, the time scale  $\tau_i$  for ionisation relaxation within the low-temperature plasma is estimated to be  $3 \times 10^4/n_e$  yr, where  $n_e$  ( $\text{cm}^{-3}$ ) is the post-shock electron density and has been estimated<sup>4</sup> to be about  $8 \text{ cm}^{-3}$ ; it can readily be shown that this value for  $n_e$  assures approximate pressure balance<sup>5</sup> between the low-temperature plasma and optical filaments<sup>6,7</sup>. We may then set  $\tau_i \approx 4 \times 10^3$  yr for the low-temperature plasma. On the other hand, a sure upper limit to the remnant's age,  $\tau$ , can be obtained from the inequality,  $\tau < r/v_b$ , where  $r$  is the radius of Pup A which may be set equal to  $7.3d$  pc (ref. 4),  $d$  (kpc) being the distance of Pup A, and  $v_b$  is the shock velocity of the blast wave propagating through the local intercloud medium with a density  $n_0$  of about  $0.1 \text{ H atom cm}^{-3}$  or less<sup>4</sup>. Assuming approximate pressure balance between the low-temperature plasma and the blast wave, we may write  $v_b \approx v_s(n_e/4n_0)^{1/2}$  (ref. 5), where  $v_s$  is the velocity of the shock producing the low-temperature plasma and is about  $4.5 \times 10^7 \text{ cm s}^{-1}$ . Thus, we obtain  $\tau < 4 \times 10^3 (n_0/0.1)^{1/2} d$  yr. Since  $n_0 \leq 0.1 \text{ H atom cm}^{-3}$  and  $d = 1.2\text{--}2.2$  kpc (ref. 4), the condition for ionisation equilibrium,  $\tau_i \ll \tau$ , can hardly hold within the low-temperature plasma. A similar consideration for the high-temperature plasma which has a temperature of  $13 \times 10^6$  K also leads to the same conclusion. Therefore, it seems more useful to examine a nonequilibrium ionisation model.

Recent calculations<sup>8</sup> of the time-dependent ionisation and X-ray emission of supernova remnants have shown that the nonequilibrium state of ionisation within the X-ray source results in an enhancement of the X-ray emission in the energy range below 1 keV, causing a two-component appearance of the X-ray spectrum. Although this result was obtained by assuming a uniform interstellar medium, it will hold qualitatively in the

case of Pup A where the dominant X-ray source may be a shock-heated interstellar cloud<sup>4</sup>. The nonequilibrium ionisation model will predict much less [Fe XIV] emission than the equilibrium model, since in the former the X-ray emission in the sub-keV range is not ascribed to a plasma favourable to the [Fe XIV] emission.

We now evaluate the [Fe XIV] luminosity of the shock wave responsible for the X-ray emission from Pup A. After passing through the shock front, the electrons are rapidly heated and successively ionise the ions to higher stages<sup>8</sup>. The electron temperature of about  $10^7$  K inferred from the X-ray spectrum<sup>2</sup> indicates that the iron atoms would be almost stripped of their M-shell electrons under ionisation equilibrium<sup>9</sup>. Therefore, on its way to the equilibrium state, every iron atom passes through the stage of  $\text{Fe}^{+13}$ . The average time interval during which an iron atom stays in this stage is  $(n_e C)^{-1}$  seconds, where  $C$  ( $\text{cm}^3 \text{ s}^{-1}$ ) is the total coefficient for direct ionisation<sup>10</sup> and auto-ionisation<sup>11</sup> of  $\text{Fe}^{+13}$  by electron impact. Recombinations can be safely ignored in rapidly ionising plasmas. Also, because  $(n_e C)^{-1}$  is of the order of only  $10^9$  s, changes in the electron temperature and density during this time interval can be ignored. Thus, after passing through the shock front, an iron atom emits  $(n_e \alpha_e + n_p \alpha_p) h\nu/(n_e C)$  erg on the average at [Fe XIV], where  $n_p$  ( $\text{cm}^{-3}$ ) is the proton density,  $h\nu$  (erg) is the photon energy, and  $\alpha_e$  and  $\alpha_p$  ( $\text{cm}^3 \text{ s}^{-1}$ ) are the coefficients for collisional excitation by electrons<sup>12</sup> and protons, respectively; for relatively high proton temperatures relevant to our case,  $\alpha_p$  should be calculated from the cross-section data<sup>13,14</sup>. By assuming that the shock front has an area of  $10^2 S \text{ pc}^2$  and is propagating at a speed of  $10^8 v \text{ cm s}^{-1}$  into a medium with a density of  $n_1 \text{ H atom cm}^{-3}$  and an iron abundance of  $4 \times 10^{-5} A$  in number relative to hydrogen<sup>15</sup>, the [Fe XIV] luminosity of the shock wave may be written as

$$L = 1.4 \times 10^{32} A n_1 v S R \text{ erg s}^{-1} \quad (1)$$

where

$$R = 0.1 \times [\alpha_e + (n_p/n_e)\alpha_p]/C \quad (2)$$

In Pup A,  $A n_1 v$  is probably near unity<sup>1,4</sup>, while the value of  $S$  is uncertain. Referring to an estimate<sup>4</sup> that a fraction 0.1 of the total volume of Pup A is emitting in X-rays, we write  $S = 0.1^{2/3} \times 4\pi (0.73d)^2 \times s = 1.4sd^2$ , where  $s$  is a correction factor. The quantity  $R$  defined by equation (2) depends on the electron and proton temperatures in the [Fe XIV] emission region. If the electron and proton temperatures are quickly equilibrated just behind the shock front, we may adopt a single temperature of  $10^7$  K inferred from the X-ray spectrum<sup>2</sup>. In this one-fluid case,  $R = 0.81$  when  $n_p/n_e = 0.85$  (ref. 15), and equation (1) yields  $L = 1.6 \times 10^{32} A n_1 v s d^2 \text{ erg s}^{-1}$ . On the other hand, if the electrons are not heated significantly at the shock front compared with the protons, we should adopt a lower electron temperature and a higher proton temperature than  $10^7$  K since [Fe XIV] emission originates nearer to the shock front than X-ray emission. Inspection of the results of our recent calculations<sup>8</sup> gives electron temperatures of about  $5 \times 10^6$  K for the  $\text{Fe}^{+13}$  region. The proton temperature need not be evaluated accurately, since  $\alpha_p$  attains a maximum value of  $4.1 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1}$  at a temperature of  $3.2 \times 10^7$  K and is very insensitive to the proton temperature in the range  $(1\text{--}10) \times 10^7$  K. We set  $\alpha_p = 4 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1}$ . In this two-fluid case,  $R = 1.9$ , and equation (1) yields  $L = 3.7 \times 10^{32} A n_1 v s d^2 \text{ erg s}^{-1}$ . Thus, in both the one-fluid

case and the two-fluid case, the nonequilibrium ionisation model predicts a [Fe XIV] luminosity that is of the same order as the observed<sup>1,16</sup> values of  $(1-5) \times 10^{32} \text{ d}^2 \text{ erg s}^{-1}$ . Also, a similar calculation of [Fe X] 6,374 Å emission predicts that the [Fe X]/[Fe XIV] luminosity ratio is 0.14 in the one-fluid case or 0.098 in the two-fluid case, in accordance with an upper limit of 0.3 derived from the observation<sup>1</sup>.

Thus the discrepancy found by Lucke *et al.*<sup>1</sup> between the X-ray and [Fe XIV] data on Pup A can be removed by the nonequilibrium ionisation model for the X-ray emission from this object. Note that the bulk of the observed [Fe XIV] emission need not be ascribed to a rapidly ionising plasma behind the shock front of the X-ray source. For instance, the brightest [Fe XIV] emission region observed near the edge of the radio shell seems, because of its high surface brightness, to have been produced by the interaction of the shock front with a density inhomogeneity<sup>1</sup>, which contributes little to the X-ray emission. If that is the case, the [Fe XIV] emission from this region may be analysed under the assumption of ionisation equilibrium, since at the post-shock temperature of about  $3 \times 10^6 \text{ K}$  the ionisation state of iron relaxes to the equilibrium much faster than that of oxygen<sup>17</sup>. Future observations with better spatial and spectral resolution will be important in clarifying the nature of the [Fe XIV] emission from Pup A.

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## Hydrogen evolution from water induced by visible light mediated by redox catalysis

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Noble metal dispersions are suitable for mediating light-induced hydrogen<sup>1-5</sup> and oxygen<sup>6-8</sup> evolution from water. We report here a dramatic improvement of the hydrogen production rate when very finely dispersed platinum is used as a mediator in reaction (1). The first observations of the dynamics of intervention of the Pt particles in the redox events are presented. A centrifuged colloidal Pt catalyst stabilised by polyvinyl alcohol showed exceptionally high activity in promoting hydrogen evolution from water via



where  $\text{MV}^+$  stands for reduced methylviologen. The latter is produced photochemically in aqueous solution containing  $\text{Ru}(\text{bipy})_3^{2+}$  as a sensitizer and EDTA as an electron donor. At  $10^{-3} \text{ M}$  Pt the reoxidation of  $\text{MV}^+$  requires only 15  $\mu\text{s}$  and leads to quantitative formation of  $\text{H}_2$ .

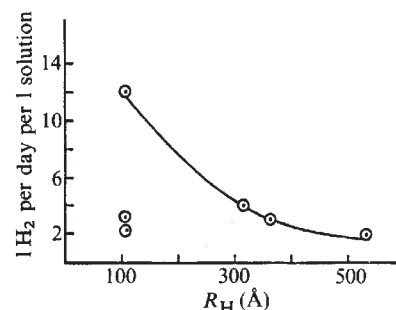


Fig. 1 Correlation for the observed yields for  $\text{H}_2$  in the system  $\text{Ru}(\text{bipy})_3^{2+} 4 \times 10^{-3} \text{ M}$ ,  $\text{MV}^{2+} 2 \times 10^{-3} \text{ M}$ , EDTA  $3 \times 10^{-2} \text{ M}$  at pH 5 and the radius of the catalyst particles used. Pt-PVA-60,000 catalysts centrifuged at 20,000 r.p.m. were used containing 3.5 mg Pt per  $25 \text{ cm}^3$  of solution.

The Pt/PVA catalyst was prepared by a procedure outlined by Nord<sup>9</sup>. By centrifuging this preparation at different speeds and for different times particle sizes between 530 and 110 Å are determined by quasi-elastic light scattering. The polymer PVA molecules are adsorbed on the surface of Pt metal through their less polar groups. The polymer also hydrates strongly in the presence of water due to its own OH groups. As long as the surface coverage is not complete,  $\text{H}_2\text{O}$  can penetrate to the Pt and the catalysis is mediated by a lyophobic system. In the case of particle size with radius 110 Å the size of the PVA particle on which the Pt is deposited is equal to the polymer PVA-60,000 indicating that there is a good dispersion of Pt on the polymer.

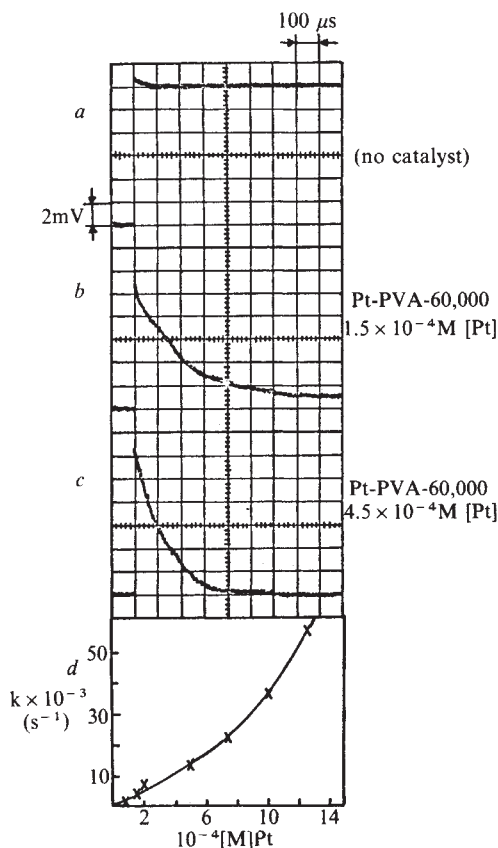
Steady-state irradiations were carried out with a 450 W xenon lamp through 400 nm cut-off filter and a water filter to eliminate IR radiation. Hydrogen was analysed by conventional GC with a molecular sieve 5 Å column. A detailed account of the light-induced events leading to irreversible reduction of (1'-dimethyl-4,4'-bipyridinium)<sup>2+</sup>, abbreviated to  $\text{MV}^{2+}$  to  $\text{MV}^+$  and oxidation of EDTA has been given elsewhere<sup>3,4</sup>. In the presence of colloidal Pt,  $\text{MV}^+$  reoxidises with simultaneous evolution of  $\text{H}_2$ .

Figure 1 shows that a decrease of the particle radius from 500 to 100 Å leads to a drastic increase in the hydrogen evolution rate which is as high as 12 l per day per l solution for the smallest particle size. In the latter case, the bubbling of hydrogen gas which occurs when the solution is illuminated is readily seen. In Fig. 1 the common feature is the Pt content of the solutions of different particle size, namely 3.5 mg Pt per  $25 \text{ cm}^3$ . Figure 1 also shows two extra points with 100 Å particle size but with low Pt levels; 0.35 mg Pt per  $25 \text{ cm}^3$  solution, the upper one, and 0.25 mg Pt per  $25 \text{ cm}^3$  solution, the lower one. The yields of  $\text{H}_2$  are 5 and 6 times, respectively, less than if 3.5 mg Pt per  $25 \text{ cm}^3$  solution is photolysed as shown in Fig. 1.

These observed size effects may be explained by the fact that the Pt particles intervene as microelectrodes<sup>10-12</sup> in the hydrogen evolution reaction (1). Electron transfer from  $\text{MV}^+$  to the Pt particles regulates these microelectrodes cathodically until  $\text{H}_2$  formation can take place. A smaller electrode size is advantageous both from the point of view of mass transport of the electroactive species as well as surface area per g of catalyst used. Note that in our experimental conditions the speed of electron turnover in the  $\text{MV}^+/\text{MV}^{2+}$  relay—and not the photon flux or the kinetics of  $\text{MV}^{2+}$  photoreduction—is rate determining in the hydrogen generation process. The quantum yield for  $\text{H}_2$  formation was found to be half of that for  $\text{MV}^{2+}$  reduction,  $\phi(\text{H}_2) = 0.13$ . This implies that the reoxidation of  $\text{MV}^+$  strictly follows the stoichiometry indicated in equation (1).

A further advantage of the centrifuged Pt/PVA is that the solutions remain completely transparent even at high catalyst concentration. This allows the direct study of the dynamics of the reaction of  $\text{MV}^+$  with Pt particles by laser photolysis. Figure 2a-c illustrates the temporal behaviour of the characteristic





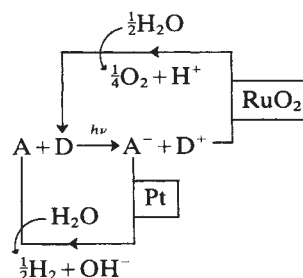
**Fig. 2** Effect of the Pt-PVA catalyst concentration on the behaviour of  $MV^+$  absorption at 600 nm. Representative oscilloscope traces are included in *a*, *b*, *c*, *d*. The rate constants for  $MV^+$  decay are plotted against platinum concentration of the Pt-PVA catalyst used.

$MV^+$  absorbance at 602 nm in the absence and presence of catalyst. The upward deflection of the signal after the laser pulse is due to the formation of  $MV^+$  via the photo redox process<sup>3</sup> sensitised by  $Ru(bipy)_3^{2+}$ . In the absence of catalyst the  $MV^+$  absorption is stable, addition of colloidal Pt induces a decay of the signal the rate of which increases sharply with Pt concentration. Fitting the absorbance decay curves to an exponential time law gives the rate constants which are plotted as a function of Pt concentration in Fig. 2*d*. The curve is steeper than linear, indicating that the reaction order is greater than one with respect to the Pt concentration.

At a concentration of  $10^{-4}$  M Pt in Pt-PVA-polymer, we observe a rate constant  $k = 0.14 \times 10^3 \text{ s}^{-1}$ . At the highest concentration of catalyst ( $12.5 \times 10^{-4}$  M Pt content), the rate was  $5.7 \times 10^4 \text{ s}^{-1}$ . The lifetimes observed for  $MV^+$  were shortened from about 7,000  $\mu\text{s}$  to 15  $\mu\text{s}$ , when the concentration of catalyst varied only by a factor of about 12.

The minimum concentration of platinum which gives efficient recycling of  $MV^+$  under steady-state illumination is  $0.5 \times 10^{-4}$  M. The lowest useful concentration necessary to detect measurable quantities of photo-produced  $H_2$  is  $0.1 \times 10^{-4}$  M in Pt (0.05 mg Pt per  $25 \text{ cm}^3$ ). In this case, the evolution of  $H_2$  observed was 0.010 l  $H_2$  per day per l solution, but a noticeable amount of hydrogen gas bubbling out of solution could only be observed at 5 times the concentration of Pt necessary for the onset of  $H_2$  evolution. Evidently, such a Pt content is sufficient to reoxidise the  $MV^+$  rapidly enough to avoid a significant build up of  $MV^+$  concentration. An onset value for the amount of Pt present seems necessary to obtain reasonable rates of  $H_2$  evolution (see Fig. 1). The value for this onset is about  $0.5 \times 10^{-4}$  M Pt and the recycling at higher concentrations of Pt is shown in Fig. 2*b*, *c*.

These results are important for photo-redox splitting of water into hydrogen and oxygen:



it seems that by using a suitable catalyst, the conversion of  $A^-$  into  $A$  and simultaneous formation of hydrogen can be accomplished so rapidly that it can compete efficiently with the thermal backreaction. (In the photostationary state achieved under sunlight irradiation, the latter process, due to its bimolecular nature, should require at least several milliseconds.) Moreover, redox catalysts such as colloidal  $RuO_2$  have been shown<sup>7,8,13</sup> to mediate very effectively the back conversion of  $D^+$  into  $D$  under simultaneous production of oxygen. In fact, in a mixture of  $RuO_2$  and Pt colloids serving as selective microelectrodes for the  $A/A^-$  and  $D^+/D$  redox couples, light-induced water splitting in a two-component system has recently been observed<sup>13</sup>.

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## Detection of elastic strain in an amorphous polymer by X-ray scattering

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For crystalline solids, the displacement of diffraction peaks with applied elastic strain is the basis of one method of measuring internal stress<sup>1,2</sup>. The effect has been exploited for both metals<sup>3</sup> and crystalline polymers<sup>4</sup>. Hitherto, the diffuse character of diffraction peaks obtained from non-crystalline materials has discouraged similar measurements on them. Working with atactic polymethyl methacrylate (a-PMMA) as an example of non-crystalline polymer glasses (which can sustain elastic strains up to several per cent before plastic yield or fracture), a displacement of the most intense diffraction peak has been observed in response to applied elastic strain. This peak corresponds to intermolecular correlations in the polymer<sup>5,6</sup>.

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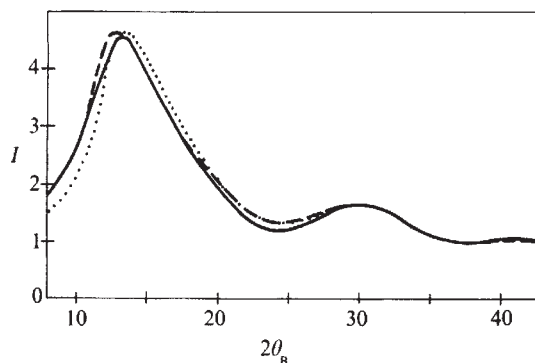
X-ray diffraction scans from a-PMMA show three broad peaks of heights which decrease in order of increasing diffraction angle. Using  $\text{CuK}\alpha$  radiation, the maxima are at  $13^\circ$ ,  $30^\circ$  and  $42^\circ$   $2\theta_B$ . (These angles correspond to Bragg spacings of 6.8, 3.0 and  $2.2\text{\AA}$  respectively, which, of course, for a non-crystalline material have limited meaning.) Orientation of the PMMA glass by plastic deformation demonstrates that the major component of the first peak is equatorial and represents intermolecular correlations, whereas the  $30^\circ$  and  $42^\circ$  peaks concentrate on the meridian and are intramolecular in origin<sup>5,6</sup>.

Dumbbell shaped tensile specimens, cut from 1 mm thick sheet (ICI Perspex), were stressed at room temperature and held at constant elastic strain. The maximum extension obtained before fracture was  $\sim 11\%$ . Diffraction scans of the strained specimens were obtained using a standard transmission diffractometer. Examples for a specimen strained to  $\sim 8.8\%$ , with the scattering vector first parallel to and then perpendicular to the tensile axis, are shown in Fig. 1. Also included for comparison is the diffraction scan for an undeformed specimen. As can be seen, the principal effect is a strain-induced displacement of the first peak. It moves to a lower angle when the scattering vector is parallel to the tensile axis, and to a higher angle when the vector is normal to the axis.

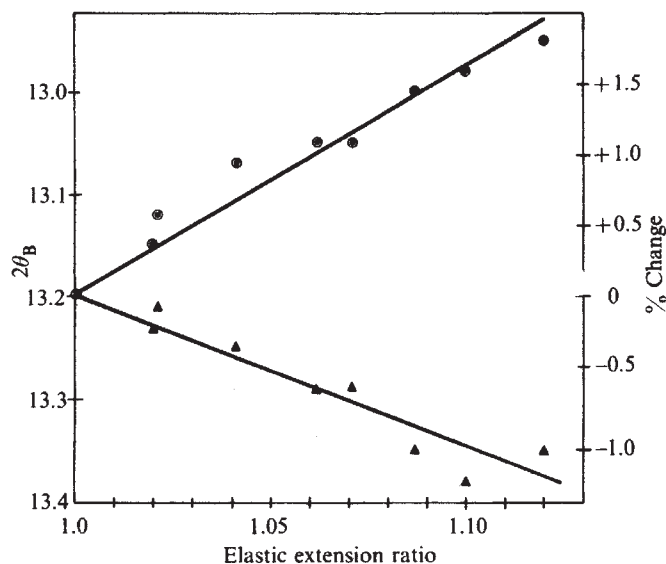
The fact that the positions and intensities of the second and third peaks, which are sensitive to molecular conformation<sup>6</sup>, are not significantly affected by elastic strain indicates that for this polymer the underlying conformation does not change in response to the applied stress. There is also no suggestion of orientation of the molecular axes as a result of elastic strain, although this commences once the polymer yields and leads to an intensification of the second and third diffuse peaks on the meridian<sup>7</sup>.

Figure 2 shows the displacement of the first peak as a function of applied elastic strain. The right hand ordinate expresses the percentage change in interchain spacing as determined by the change in  $\text{cosec } \theta_B$ .

For a crystalline solid the most appropriate simple model would be one in which the material deforms affinely, that is the chain segments move as though embedded in a continuum. Such a model predicts a change in the interchain spacing equal to the applied strain. However, for PMMA the X-ray measurements of the microscopic strain give values which, although in the same sense, are much smaller than the strains applied. A tensile strain of 10% produces an increase in interchain spacing of only 1.7%, whereas the associated lateral compressive strain which, for PMMA with a poissonian ratio of 0.39, will be 3.9% apparently corresponds to a decrease in interchain spacing of 1.1%. The microscopic strains are thus, 17% and 28% respectively, of the applied strain.



**Fig. 1** Comparison of diffracted intensities for scans: dashed line, scattering vector parallel to the applied tensile stress; dotted line, perpendicular to the stress; solid line, for the unstressed specimen. The applied tensile elastic strain was 8.8%.



**Fig. 2** The change in position of the first broad diffraction peak as a function of elastic strain. ○, Scattering vector parallel to the applied tensile stress; ▲, perpendicular. The right-hand ordinate is calibrated in terms of the change in apparent chain spacing.

The other simple model is one in which the microscopic strain is solely due to the increase in volume of the material (caused by the hydrostatic component of the tensile strain). While this predicts a change in interchain spacing of approximately the correct amount (10% tensile strain giving  $\sim 1\%$  increase in interchain spacing for PMMA), it is unable to account for the fact that the change normal to the tensile axis is a decrease.

These discrepancies suggest that a more appropriate model may be a non-affine one in which the distance between nearest-neighbour chains remains essentially constant. For PMMA, which has a fairly rigid backbone, such contacts may involve some correlation of segmental orientation. However, even without such correlation, the distance between nearest-neighbours initially in contact is likely to change less under stress than will the distance between near-neighbours which are not in contact. Since it is the nearest-neighbour contacts which have a dominant influence on the position of the intermolecular diffraction peak, such a model would explain the reduced sensitivity of peak position to applied strain. At the same time, the volume increase and macroscopic strain would be accounted for by a change in the number and arrangement of the near-neighbour chains.

The fact that there is still no satisfactory model to describe molecular packing in non-crystalline polymers means that these arguments cannot be developed further and quantified. Also, any detailed understanding will have to take into account the thermally activated (anelastic) component of the applied strain which is associated with changes of local molecular orientation, or of sidegroup geometry, which themselves will influence packing.

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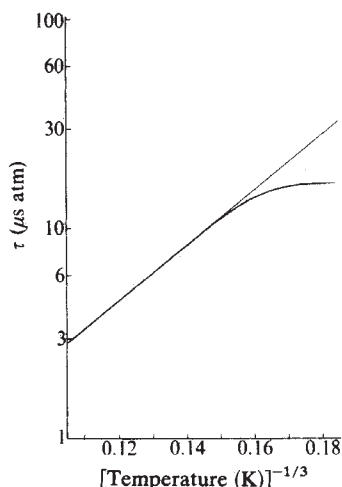
## Radiative cooling near the mesopause

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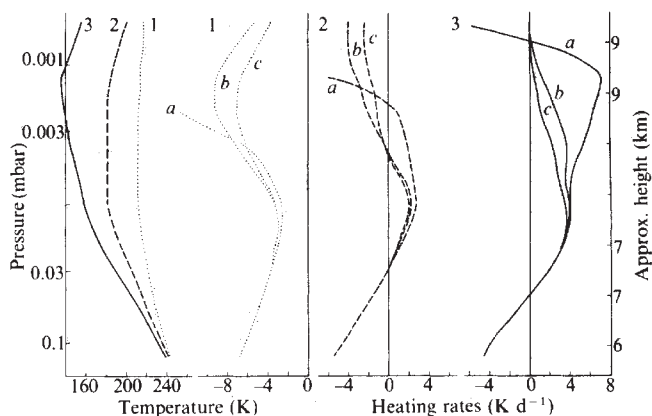
The heat budget of the region of the upper atmosphere near the mesopause at  $\sim 85$  km is determined by a balance between radiative, photochemical and dynamical effects leading to a very cold polar mesopause in the summer and a comparatively warm mesopause in the winter. There is a temperature minimum at the mesopause primarily because of the strong radiative cooling which occurs due to thermal emission by carbon dioxide in its  $\nu_2$  vibrational band at  $15\ \mu\text{m}$  wavelength. Above 80 km this band is no longer in local thermodynamic equilibrium (LTE) so the amount of cooling depends critically on the rate at which  $\text{CO}_2$  molecules are excited or relaxed by collision. Any estimate of radiative cooling for this region of the atmosphere, therefore, relies on knowledge of the collisional relaxation time,  $\tau$ . The importance of the temperature dependence of  $\tau$  was pointed out by Houghton<sup>1</sup>. Previous calculations used a wide range of values because no direct measurements of  $\tau$  had been made in the appropriate temperature range. We show here that the effect on cooling rate calculations of using values of  $\tau$  measured at temperatures down to 175 K. We also estimate a radiative relaxation time for the atmosphere near the mesopause and are able to draw conclusions as to the natural lifetime of any temperature perturbation at this altitude.



**Fig. 1** The relaxation time of the  $\nu_2$  vibration of  $\text{CO}_2$  due to  $\text{CO}_2\text{-N}_2$  collisions. The thin line shows a linear extrapolation of the higher temperature measurement.

The  $\text{CO}_2$  molecules may be excited into the  $\nu_2$  state by the  $\text{N}_2$ ,  $\text{O}_2$  and O atoms present. Water is a very effective collision partner, but in the upper atmosphere its effect is negligible owing to its very low concentration. In previous calculations of cooling rates<sup>1-4</sup>, values of the relaxation time,  $\tau$ , at 1 atm between 2 and 50  $\mu\text{s}$  have been used.

The reason for this spread was because of the difficulties in making reliable estimates of  $\tau$ . A scatter of at least  $\pm 25\%$  existed in the older data at 300 K<sup>4</sup> and it was not known whether the nearly linear plots of  $\log \tau$  against  $T^{-1/3}$  could be extrapolated down to 180 K. For instance, on the basis of data for the

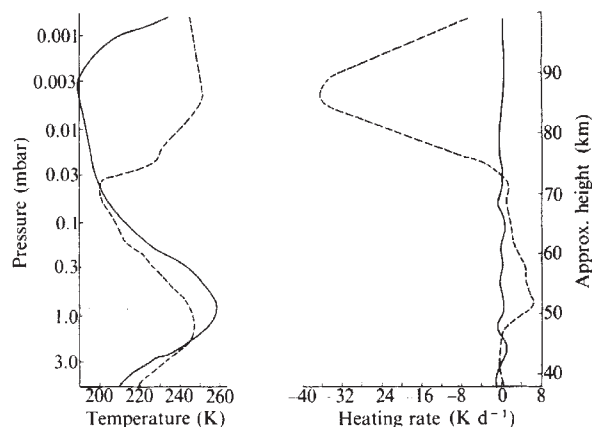


**Fig. 2** Temperature profiles for (1) January at latitude  $70^\circ\text{N}$ ; (2) USA 1962 Standard Atmosphere; (3) December at latitude  $70^\circ\text{S}$ . And the corresponding heating rates assuming (a) local thermodynamic equilibrium, (b) the new measurements of  $\tau$ , (c)  $\tau$  values from extrapolation of measurements at higher temperatures.

relaxation time of  $\text{CO}_2$  by  $\text{CO}_2$  and by Ar taken down to 150 K<sup>6</sup>, perhaps the best estimate of the relaxation time of  $\text{CO}_2$  by air at 210 K to be made up to the present was 22  $\mu\text{s}$  (ref. 7).

Figure 1 shows the results of recent laser fluorescence measurements of the relaxation time of the  $\nu_2$  vibration of  $\text{CO}_2$  due to  $\text{CO}_2\text{-N}_2$  collisions. Further details of these measurements are given by Allen<sup>8</sup>. They are the first direct measurements over the range of temperature appropriate to the mesopause region, and show much less dependence on temperature at temperatures below 250 K than would be expected from an extrapolation of the higher temperature data. On the basis of the work of Simpson *et al.*<sup>7</sup> we anticipate that the relaxation time for  $\text{CO}_2$  by  $\text{O}_2$  will be about 20% faster than for  $\text{CO}_2$  by  $\text{N}_2$ . For air at 180 K this gives  $\tau = 15 \pm 2\ \mu\text{s}$  at 1 atm, that is, 30% less than the previous best estimate.

In Fig. 2 the effects of different values of  $\tau$  on the radiative divergence near the mesopause are calculated using the Curtis matrix method as described by Williams<sup>4</sup> and Houghton<sup>9</sup>. This method allows for transfer of radiation between different levels of the atmosphere and for the breakdown of local thermodynamic equilibrium: the transmission function incorporates the Curtis-Godson approximation, a constant diffusivity factor and a band model of independent lines. Note that an additional radiative cooling of  $\sim 2\ \text{K d}^{-1}$  arises from the difference between



**Fig. 3** Solid lines show approximate radiative equilibrium temperature profile calculated for latitude  $30^\circ\text{N}$  in January and its corresponding heating rate. Dashed lines show an extreme temperature profile measured from a rocket<sup>8</sup> and its corresponding heating rate.

using straight extrapolation and using the latest values. In the absence of other heating mechanisms this would have to be balanced by dynamical heating due to an increase in downwards motion of about  $200 \text{ m d}^{-1}$ —a very significant consideration in the context of the mesospheric circulation.

Another quantity which is important in understanding the structure of the mesosphere especially in perturbed conditions is the time constant with which radiation processes tend to return a perturbed atmosphere to the mean state. Figure 3 shows a radiative equilibrium temperature profile calculated for latitude  $30^\circ \text{N}$  in January using the Curtis matrix method, with the new  $\tau$  values, and for the  $15 \mu\text{m}$   $\text{CO}_2$  cooling and an 'exact' frequency dependent calculation for the heating due to absorption of solar UV radiation by ozone and molecular oxygen. Also shown is a rather extreme temperature profile similar to one measured on 4 January 1976 by some rockets<sup>10</sup>. Other data in the same series suggest that this profile persisted for some while being part of a wavelike disturbance with period 7 or 12 days. The net radiative heating rate was calculated for the latter profile using the methods referred to above. It shows a cooling of  $36 \text{ K d}^{-1}$  where the temperature deviates by 60 K indicating that the radiative time constant at 85 km altitude is of the order of 2 days. Perturbations of the mesospheric temperature, therefore, are very strongly damped and cannot persist for periods of longer than a few days unless they are continuously forced.

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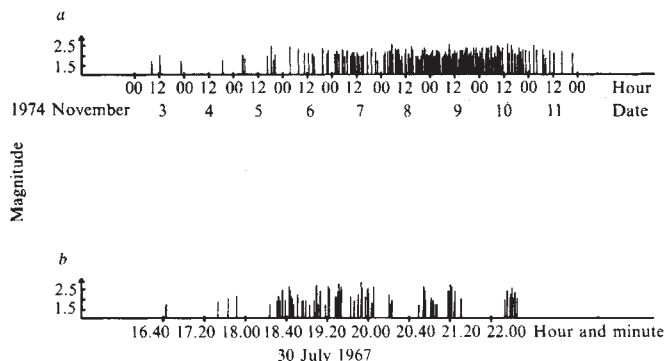
## Intraplate earthquake swarms in Greenland and adjacent continental regions

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**Earthquake swarms, consisting of many earthquakes of nearly equal magnitude within a small area, often occur in areas of recent or current volcanic or tectonic activity<sup>1</sup>. But earthquake swarms can also be recognised in some typical intraplate areas in northern Canada<sup>2</sup>, Norway and Greenland. A swarm of earthquakes started in Melsøy in November 1978, remarkable because no earthquake swarms in Norway<sup>3</sup> have been documented previously. The Melsøy swarm occurred near the Norwegian coast, on one side of the Norwegian/Greenland Sea. A few earthquake swarms have occurred also in Greenland, near the east coast, by the Norwegian/Greenland Sea, and near the north coast, by the Arctic Ocean as have most of the swarms in northern Canada<sup>2,4</sup>. Some common features of the earthquake swarms in Greenland, Norway and northern Canada are indicated here.**

The earthquake swarms in Greenland have occurred in areas with a tectonic setting and an earthquake activity similar to that of Norway. Figure 1 shows two time sequences of earthquakes within small areas. As none of the earthquakes is the main event in either sequence, the sequences are termed earthquake swarms. The largest earthquakes of the swarm in eastern



**Fig. 1** Time sequences of the earthquake swarms in eastern (a) and northern (b) Greenland. Magnitudes are based only on readings from the seismological stations DAG and ALE respectively. No earthquakes occur at the swarm locations either immediately before or after the illustrated time intervals.

Greenland had magnitude 2.7 according to the Nuttli scale<sup>5</sup>. The smallest earthquakes of the swarm, which can be identified as having occurred in the same source area, had magnitude 1.9. A simple linear relationship between the magnitude and the logarithm of the number of earthquakes in each magnitude range could not be determined reliably—no b-value could be found for the earthquake swarm. Altogether the energy release in the swarm corresponds to the energy release of one single earthquake of magnitude a little over 3 (a small event).

The earthquake swarm in northern Greenland lasted a shorter time than that in eastern Greenland. (The time scales of Fig. 1 a and b are different; 2 min for the sequence in northern Greenland correspond to 1 h for the sequence in eastern Greenland.)

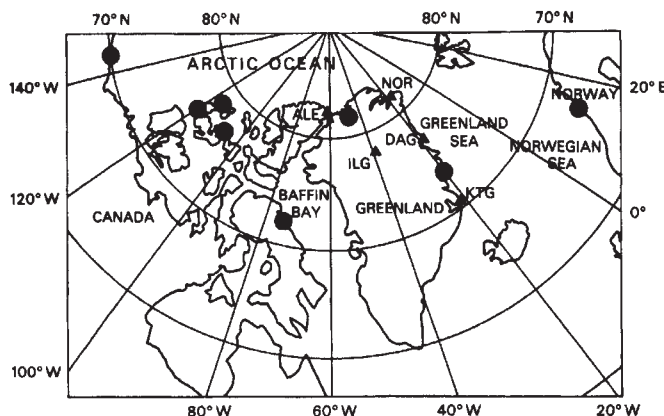
The largest earthquake of the swarm in northern Greenland had magnitude 2.9 according to the Nuttli scale<sup>5</sup>. The smallest earthquakes, which can be clearly identified to have happened in the same source area, had magnitudes 1.8. The b-value for the earthquake swarm is around 1.0 with a large uncertainty, because the magnitude range is small. The energy release in the swarm corresponds to the energy release of a single earthquake of magnitude a little over 3, which is a small event and similar to that in eastern Greenland. In the swarm in eastern Greenland ~160 earthquakes have been distinguished, while in the swarm in northern Greenland 80 earthquakes have been found.

In eastern Greenland the earthquake swarm location around  $73.5^\circ \text{N } 20^\circ \text{W}$  (Fig. 2) is based on seismograph records of the 25 largest earthquakes of the swarm from two seismological stations in Danmarkshavn (DAG) and in Kap Tobin (KTG). The uncertainty in the location of the swarm is 100 km in an east-west direction, while it is 25 km in a north-south direction. The earthquake swarm is in an area where neither single earthquakes nor earthquake sequences are known to have happened previously. The smaller earthquakes of the swarm have the same recorded signature at station Danmarkshavn (DAG) as the larger earthquakes, but they cannot be distinguished at the less sensitive station Kap Tobin (KTG). None of the earthquakes were recorded at other seismological stations.

The calculation of the location of the earthquake source area in northern Greenland  $81.5^\circ \text{N } 49^\circ \text{W}$  (ref. 4) (Fig. 2) is based on records from the seismological stations Alert (ALE), Inge Lehmann (ILG) and Nord (NOR). Not all of the earthquakes could be distinguished at station Nord. Earthquake sequences have been observed at the station Alert several times before, as well as after, the sequence in Fig. 1, at similar distances, presumably near the same location. The ILG station operated for only 1 yr and only the abovementioned earthquake sequence occurred during that time. The earthquakes were not recorded at other seismological stations.

In northern Canada earthquake swarms are quite common<sup>2</sup>. All of the swarm locations there are plotted in Fig. 2. In Canada, Greenland and Norway the swarms are situated a long way from





**Fig. 2** Map with the recognised intraplate earthquake swarms (●). For the continental regions adjacent to Greenland the locations of the earthquake swarms are from published sources<sup>2-4,6-10</sup>. ▲, Seismological stations, mentioned in the text, with a three-letter code.

present plate boundaries. The swarms in Norway and in eastern Greenland are close to the passive continental margins of the Norwegian/Greenland Sea, in the middle of which an active mid-ocean ridge is located<sup>11</sup>. The swarms in northern Greenland and northern Canada<sup>2,4</sup> are located close to the passive margins of the Arctic Ocean and Baffin Bay. In the Arctic Ocean there is an active mid-ocean ridge<sup>2</sup>. In Baffin Bay there is no active mid-ocean ridge; the active spreading ceased >40 Myr ago<sup>12</sup>. In all of the swarm locations there are basins of sediments close to the continental margins<sup>3,4,13,14</sup>. The swarm activities are close to these sediment basins, whether these are of Palaeozoic ages as in northern Greenland or of Mesozoic ages as in eastern Greenland. These swarm locations are at the edges of continental areas which since the end of the last ice age (~10,000 yr ago) have been exposed to considerable uplift. The earthquakes in the swarms are found to be shallow in the cases where they could be determined<sup>3,4,6</sup> so the earthquake swarms presumably occur in the upper crust.

The swarms did not occur in the usual locations of earthquake swarm activity, which are areas of current or recent volcanic or tectonic activity<sup>1</sup>. They occurred far from interplate boundaries, so they may be classified as intraplate earthquake swarms. Sykes<sup>15</sup> noted that many intraplate earthquakes occur along old zones of weakness near continental margins and the same interpretation is reached from the present results. The earthquake swarms occurred along continental margins where the structures in and around the old sedimentary basins are the zones of weakness. The geological ages of the sedimentary basins are of little importance. The tectonic stresses which give rise to the intraplate earthquakes may be those connected with the present plate tectonics motion<sup>15</sup>, but a special source of stresses in Greenland and the adjacent continental regions is the considerable uplift of these regions since the last ice age.

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## Radiocarbon chronology of late Pleistocene Konya lake, Turkey

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The Konya basin in south central Turkey, which is now largely dry, was formerly occupied by an extensive lake<sup>1,2</sup>. Although the lake is generally considered to be of glacial age, its precise chronology has remained uncertain<sup>3</sup>. We report here preliminary <sup>14</sup>C dates on shells which suggest that lake level attained its last maximum 23,000-17,000 yr ago. We further propose that its subsequent retreat reflects a late glacial climate that was dry as well as cold.

The Konya plain is an area of internal drainage lying ~1,000 m above m.s.l. on the southern edge of the Anatolian plateau (Fig. 1). Rivers enter the basin from the Toros (Taurus) mountains to the south and west, and eventually discharge into small lakes at Ak gölü and Hotamis and marshes at Aslim and Hamidiye. The rest of the plain is now dry. Shorelines of the former lake can easily be traced from eroded cliff-lines and depositional features such as beaches and spits, which do not seem to have been subsequently affected by folding or faulting. The lake covered ~4,340 km<sup>2</sup> at its last maximum, but the depth of water did not exceed 30 m and at most places was <15 m. The Konya lake was thus larger but much shallower than the contemporary Lisan Lake in the Jordan rift<sup>4</sup>.

Most of the <sup>14</sup>C samples discussed here came from shell beds in sand and gravel deposits of shoreline features. The Main Series of dates were from samples taken at elevations between 1,004 and 1,012 m, and those in the Upper and Lower Series were on samples from higher and lower elevations respectively (Table 1). All dates were determined on shell, and in all but two cases the species involved was *Dreissena*, a small aragonitic bivalve which prefers fresh or brackish, open-water conditions<sup>5</sup>. The two exceptions, which date a minor lake transgression (Lower Series), were on *Lymnaea*, which was one element in a molluscan assemblage indicative of shallow, stagnant water<sup>5,6</sup>.

Contamination by young carbon is the major problem in the <sup>14</sup>C dating of shells older than 10,000 yr (ref. 7). Several shells in the Konya samples were found to have a secondary coating of calcite which had formed by percolating meteoric water after the retreat of the lake. The distorting effect of this on radiocarbon age is seen clearly in a sample from Dervişin Hanı, where the inner and outer shell fractions gave ages of 17,610±160 and 11,540±120 yr BP respectively, the latter age having been depressed by the secondary calcite cement (Table 1). One of the Kilbasan samples (10.a.i.) was also found to contain up to 8% calcite, and its age of 14,660±155 yr BP should therefore be considered as a minimum.

Other samples were analysed by X-ray diffraction and optical and scanning electron microscopy, and were hand-sorted to remove contaminated specimens before submission to the radiocarbon laboratory. This procedure allowed elimination of secondary calcite at levels above 1-2%. The resulting improvement can be seen in sample 2.a.i., also from Kilbasan, where the discrepancy between the ages of inner and outer shell fractions was greatly reduced. But because of these problems and of possible uptake of 'old' carbon, it is felt that a 10% margin of error should be applied to most of the <sup>14</sup>C dates listed here.

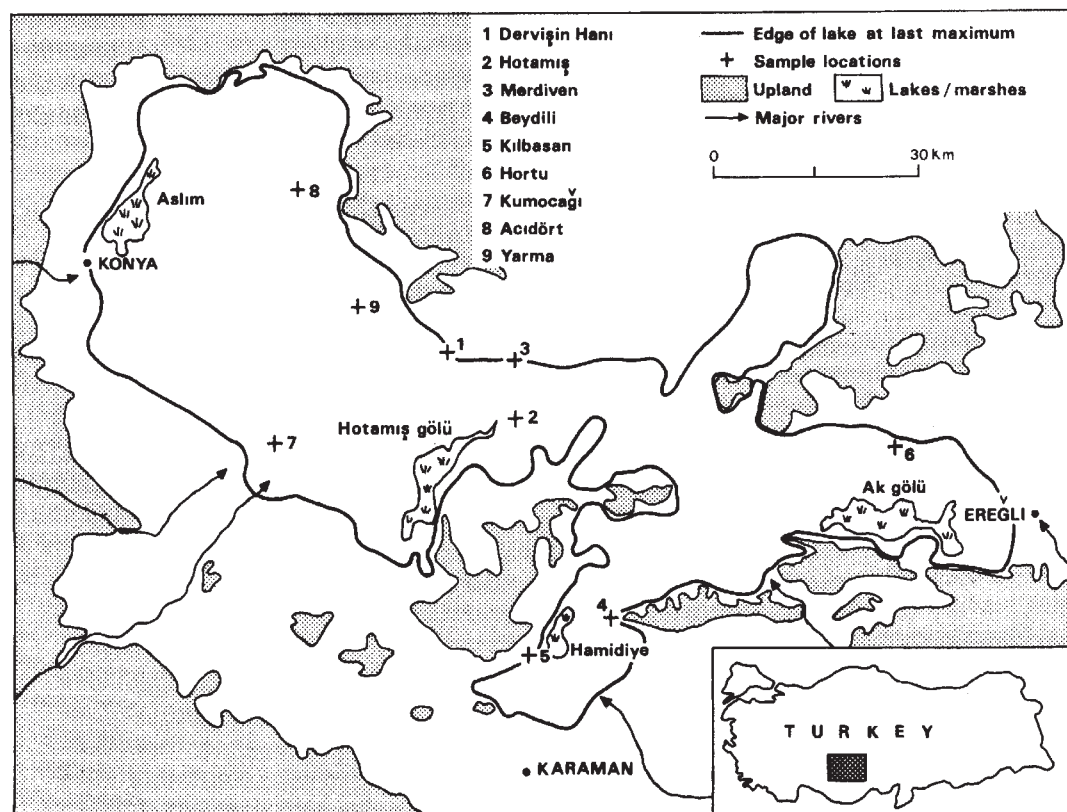


Fig. 1 Location of sample sites in Konya basin.

The radiocarbon dates are, nonetheless, consistent in suggesting that the last major phase of high lake levels in the Konya basin occurred between 23,000 and 17,000 yr BP. The lake had existed in the period immediately before this, but was at a low level. On the other hand, after 17,000 yr BP during the late glacial stage, the basin seems to have been largely dry. A minor re-advance in isolated secondary depressions between 12,000 and 11,000 yr BP may have been caused by glacial meltwater and by overflows from a higher lake system in the Beyşehir-Suğla basin. Archaeological mounds or *hüyüks* lie on the floor of the Konya basin and thus post-date the last lacustral interval. The oldest excavated *hüyüks* are Can Hasan III (with  $^{14}\text{C}$  dates back to 8,600 yr BP)<sup>8</sup> and Çatal *hüyük* (dated between 8,200 and

7,500 yr BP)<sup>9</sup>. The fauna from Can Hasan III indicates generally dry, steppic conditions in the Karaman sub-basin around 8,500 yr BP (S. Payne, personal communication). The Konya basin has therefore been dry for most of the Holocene, although there is evidence for more extensive and permanent flooding in the Konya-Çumra area during the mid-Holocene<sup>3</sup>.

The proposed late Pleistocene lake level sequence from Konya is in general agreement with those from other Middle Eastern basins, such as Lakes Lisan<sup>4</sup> and Mundafan<sup>10</sup>, where the last high-stands occurred before  $16\text{--}17 \times 10^3$  yr ago. It also differs markedly from the sequence in the south-west US, where many palaeo-lakes were at high levels between 17,000 and 10,000 yr BP (ref. 11). The early Holocene lacustral episode well

Table 1 Radiocarbon dates for the Konya lake

| Field site      | Location           | Laboratory no. | $^{14}\text{C}$ age (yr BP)    | Sample no. and comments                  |
|-----------------|--------------------|----------------|--------------------------------|------------------------------------------|
| Upper Series    |                    |                |                                |                                          |
| Merdiven        | 37°44' N, 33°13' E | GrN 6015       | 32,350 ± 410                   |                                          |
| Main Series     |                    |                |                                |                                          |
| Kilbasan        | 37°21' N, 33°13' E | UM 1638        | 22,850 ± 370                   | 2 a.i. inner shell fraction              |
| Kilbasan        | 37°21' N, 33°13' E | UM 1637        | 20,800 ± 190                   | 2.a.i. outer shell fraction              |
| Hortu           | 37°37' N, 33°49' E | GrN 5728       | 21,800 ± 455                   | shells from white lake marl              |
| Beydili         | 37°23' N, 33°22' E | UM 1639        | 21,790 <sup>+700</sup><br>-650 | 8.b.i.                                   |
| Beydili         | 37°23' N, 33°22' E | UM 1579        | 19,510 ± 450                   | 8.b.i. (rpt.)                            |
| Kumocacı        | 37°37' N, 32°48' E | GrN 6014       | 21,370 ± 215                   |                                          |
| Hotamış         | 37°39' N, 33°11' E | GrN 5729       | 20,700 ± 450                   | shells from white lake marl              |
| Kilbasan        | 37°21' N, 33°13' E | UM 1568        | 18,915 ± 325                   | 2.b.ii.                                  |
| Kilbasan        | 37°21' N, 33°13' E | UM 1577        | 14,660 ± 155                   | 10.a.i. (minimum age)                    |
| Dervişin Hanı   | 37°45' N, 33°05' E | H 4181-3412    | 17,610 ± 160                   | Ky 1b inner shell fraction               |
| (Dervişin Hanı) | 37°45' N, 33°05' E | H 4181-3411    | 11,540 ± 120                   | Ky 1a outer shell fraction (minimum age) |
| Lower Series    |                    |                |                                |                                          |
| Yarma           | 37°48' N, 32°58' E | GrN 6016       | 12,010 ± 65                    |                                          |
| Acidört         | 37°58' N, 32°52' E | GrN 5841       | 10,950 ± 65                    | shells from dark organic marl            |

All dates were calculated using the 5,568 half life, and are normalised for  $^{13}\text{C}$  fractionation to  $\delta^{13}\text{C} = -25\%$  relative to PDB. Samples listed in brackets have suffered secondary contamination, and their  $^{14}\text{C}$  ages are taken to be minima.



documented for much of Africa<sup>12</sup> and Arabia<sup>10</sup> is not recorded in the Konya basin.

The shallowness of the Konya lake and its disappearance during the post-glacial rise in temperature both point to the critical role of evaporation in the water balance of the lake. As there is no evidence of major temperature changes associated with the main lake retreat, one must conclude that this regression was a product of climatic aridity. A decline in precipitation during the late glacial is also indicated by very low levels of arboreal pollen in cores from Söğüt and the Ghab<sup>13</sup>. It thus seems that not only the tropics but also the extra-glacial mid-latitudes were subject to major variations in precipitation during the glacial Pleistocene. These variations seem, moreover, to have been out of phase with the global palaeotemperature changes indicated by deep-sea cores<sup>14</sup>. Work now in progress is intended to relate the Konya sedimentary sequence to palynological and archaeological data, and to elucidate the palaeo-hydrology of the basin.

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## Chlorophyll and nitrate fine structure in the southeastern Bering Sea shelf break front

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Ocean fronts of considerable spatial extent and temporal persistence have been described for the shelf break region along the North American Atlantic coast<sup>1-3</sup> and along the eastern Bering Sea shelf<sup>4</sup>. Intensive fishing activity in the vicinity of the shelf break front along the mid and north Atlantic coast suggests that high biological activity is associated with the front<sup>5,6</sup>. Primary productivity and chlorophyll standing crop are enhanced along the Scotian shelf break front as a consequence of vertical transport of nutrients to the photic zone<sup>7,8</sup>. Although physical data suggest that shelf break fronts persist for periods of from weeks to years, space and time scales associated with biological activity in shelf break fronts are not well known. We report here the spatial scales and fine structure of chlorophyll and nitrate distribution in near-surface waters of the shelf break front in the southeastern Bering Sea. Data collected on several cruises during an investigation of the processes and resources of the Bering Sea shelf (PROBES) allow a first order estimate of the time scales of persistence of nutrient and phytoplankton dynamics associated with the shelf break front. Properties associated with an extensive middle shelf front inshore of the shelf break front are reported elsewhere<sup>9</sup>.

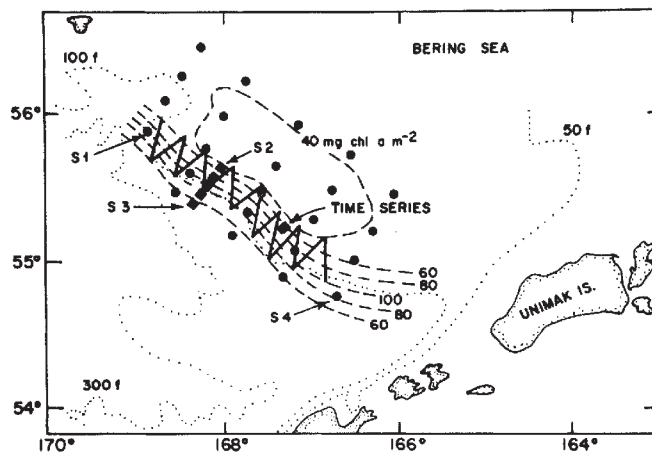


Fig. 1 PROBES study area in the southeastern Bering Sea. Contours of vertically integrated chlorophyll ( $\text{mg m}^{-2}$ ) were prepared from data taken at hydrocast stations (●). The mapping track is indicated by the solid lines. The time series station location lies near the arrowhead. S1 is station 3002, S2 is station 116, S3 is station 120, and S4 is station 3038.

CTD data taken at hydrocast stations (Fig. 1) were processed with a digital computer aboard the RV Thomas G. Thompson. The shelf break front was evident near station 120 in both density and salinity data (Fig. 2). The shelf break front is characterised by a salinity gradient which extends about 1,000 km along the continental shelf of the eastern Bering Sea<sup>4</sup>.

Chlorophyll values were obtained by *in vivo* fluorometric measurement of phytoplankton pigments in water obtained from the sea chest of the RV Thomas G. Thompson ( $z = 3 \text{ m}$ ). Continuous *in vivo* chlorophyll values measured with a fluorometer (Turner Designs) were calibrated against extracted fluorescence chlorophyll values for water samples taken every 30 min from the exhaust side of the fluorometer ( $r^2 = 0.91$ ). Nutrient concentrations were measured continuously with an Autoanalyzer for water samples taken from the sea chest line<sup>10</sup>. One-minute interval samples were taken from the detectors

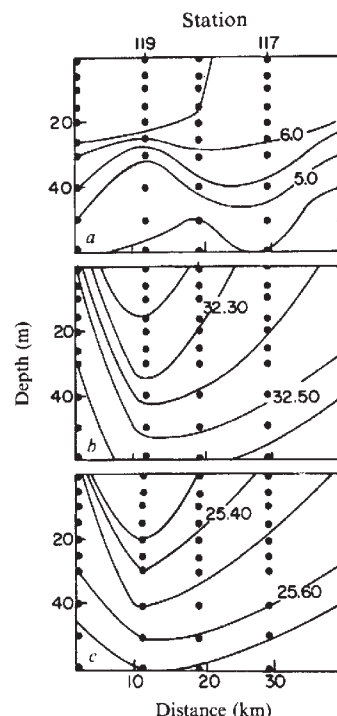
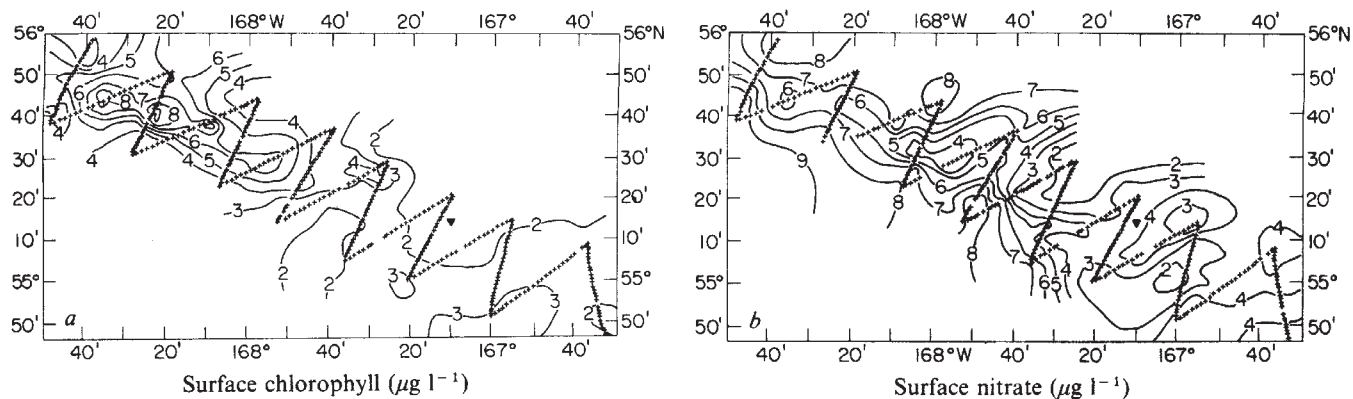


Fig. 2 Sections of *a*, temperature ( $^{\circ}\text{C}$ ); *b*, salinity ( $\text{‰}$ ); and *c*, density ( $\sigma_t$ ) across the shelf break front, 11 June 1978.



**Fig. 3** *a*, Map of chlorophyll concentration ( $\mu\text{g l}^{-1}$ ) at 3 m depth on 26 May 1978. The time series station was located at the triangle. *b*, Map of nitrate concentration ( $\mu\text{g atoms of NO}_3 \text{ per l of N}$ ) and 3 m depth on 26 May 1978.

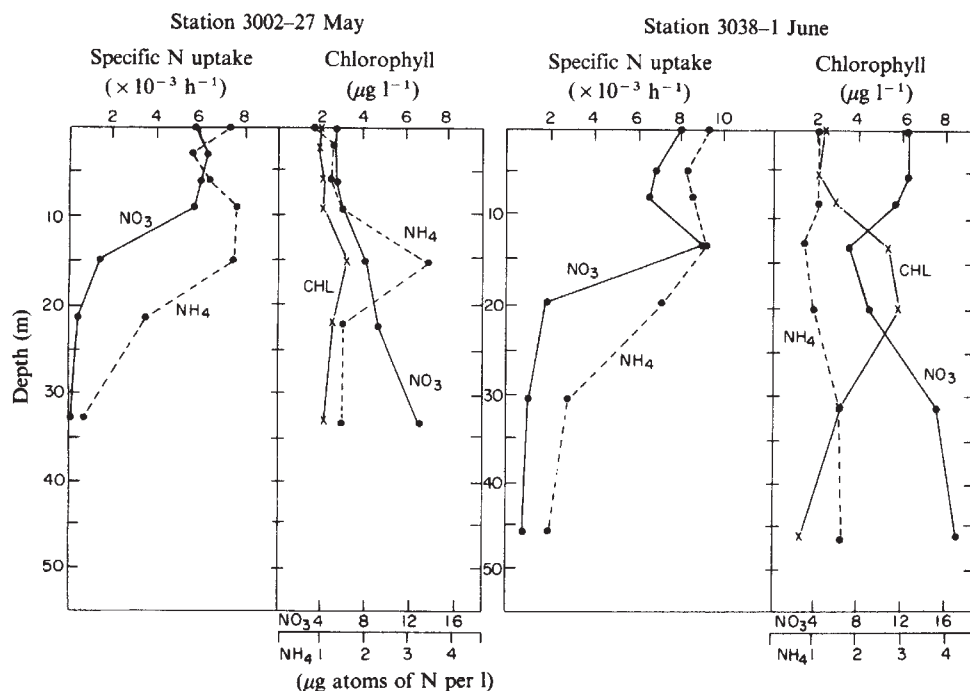
over a 36-h mapping track (Fig. 1). Five-minute averages of the samples were contoured on board ship with a digital computer to produce maps of chlorophyll and nutrient concentrations. Nutrient and chlorophyll concentrations were measured for water samples taken from Niskin bottles tripped at various depths at selected stations. Uptake velocities of nitrate and ammonium were determined for phytoplankton communities using a  $^{15}\text{N}$  isotope tracer technique<sup>11</sup>. Samples were taken periodically for microscopic examination of phytoplankton.

Vertically integrated chlorophyll values obtained from extracted fluorescence measurements for discrete water samples obtained from hydrocasts were contoured with a digital computer program. Chlorophyll was distributed along the shelf break in a band about 30 km wide with a maximum in the region of the 100 fathom isobath; integrated chlorophyll values decreased by about half on either side of the frontal maximum (Fig. 1). The front moves with the tide, with an estimated maximum speed<sup>12</sup> of  $50 \text{ cm s}^{-1}$ , which explains the difference in location of the front in the physical data (Fig. 2) and the location of the chlorophyll maximum obtained by contouring data taken at various stages of the tide.

A surface mapping track was designed to bracket the 100 fathom isobath from the Aleutian Island chain north to the Pribilof Islands. A fine structure map prepared from data collected on this track suggests the chlorophyll maximum was ~15 km wide with chlorophyll values generally larger towards

the Pribilof Islands (Fig. 3*a*). The map track covered a horizontal distance of about 170 km along the shelf break. Although the gradients across the front were evident in the chlorophyll field along the complete length of the map, structure in the near-surface chlorophyll field was most distinct from background chlorophyll in the northern 100 km. Structure in the nitrate field was not as detailed as structure in the chlorophyll field; however, local minima in nitrate concentration generally coincided with local maxima in chlorophyll concentration (Fig. 3*b*). A subsurface chlorophyll maximum present in the southern portion of the transect (Fig. 4) was not observed in the mapping data set because of the 3-m sampling depth. Silicate and phosphate concentrations did not exhibit the structure observed in the near-surface nitrate map but the general trends were similar with higher values to the north and lower values near the Aleutian Island chain.

A time series station was occupied within the shelf break chlorophyll maximum region in early May before mapping the front in late May. The vertical distribution of chlorophyll and nitrate show frontal excursion past the fixed station (Fig. 5). Surface water displacement produced a large gradient both in chlorophyll and in nitrate concentrations near the surface; however, nitrate concentrations remained relatively constant below about 40 m. The photic zone depth (depth where light intensity is 1% of the surface value) was 40 m at station 2010 outside the chlorophyll maximum region, 14 m at station 2016,



**Fig. 4** Nitrate, ammonium, and chlorophyll concentrations measured from hydrocast water samples. Specific nitrogen uptake velocities for phytoplankton from the hydrocast samples held 24 hours in a deck incubator at the light intensity at the depth where they were collected.



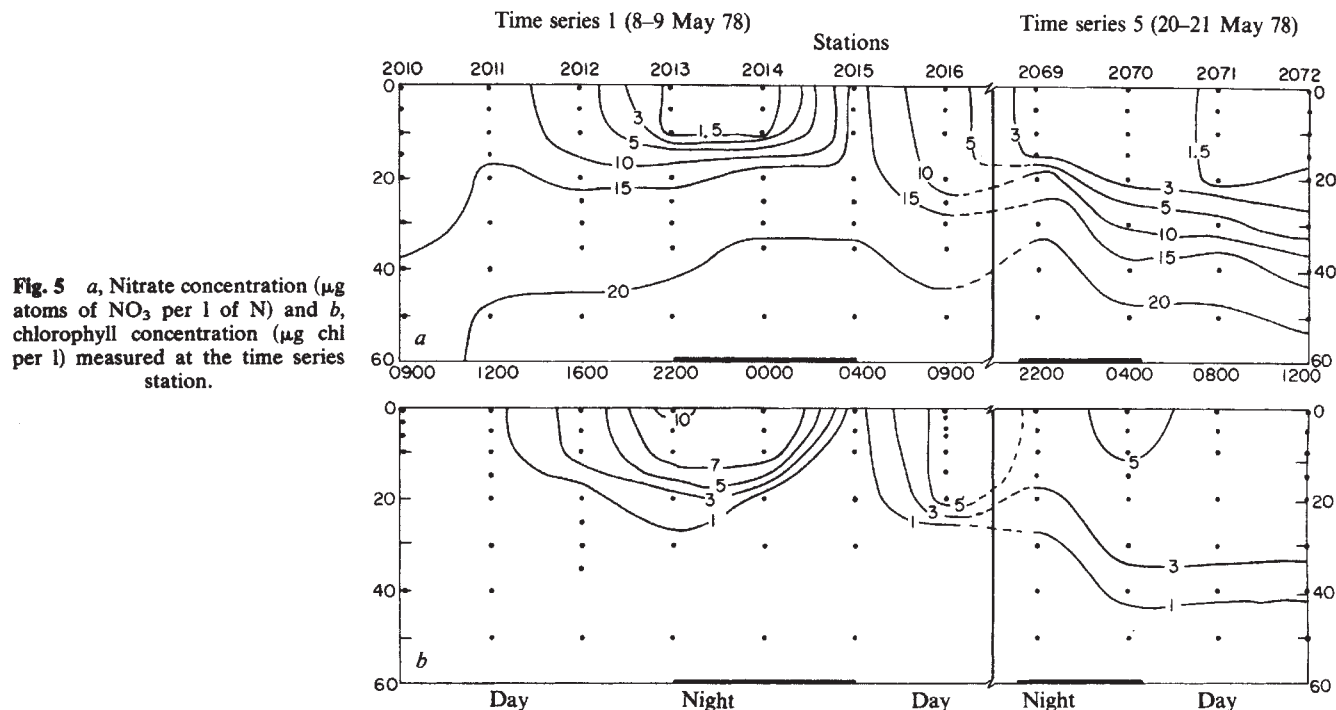


Fig. 5 a, Nitrate concentration ( $\mu\text{g}$  atoms of  $\text{NO}_3$  per l of N) and b, chlorophyll concentration ( $\mu\text{g}$  chl per l) measured at the time series station.

and 16 m at station 2071 near the chlorophyll maximum. Productivity below 20 m at station 2071 was zero for  $^{14}\text{C}$  estimates. An indication of the persistence of chlorophyll and nitrate patterns in the front with time can be seen in the continuation of the patterns in chlorophyll and nitrate concentrations with depth at the same station two weeks later. The station was located with Loran C, considered accurate to within 200 m in the southeastern Bering Sea. A significant portion of the water column chlorophyll had settled to the base of the photic zone by the end of the second phase of the time series, although vertically integrated chlorophyll values were similar at stations 2013 and 2071. Silicate was nearly constant between 28 and 30  $\mu\text{g}$  atoms of Si per l in the photic zone during the time series, supporting a contention that diatoms were not a major constituent of the phytoplankton in the shelf break front.

Nitrogen uptake velocities obtained with  $^{15}\text{N}$  nitrate and ammonium allow estimation of nitrogen doubling times for phytoplankton communities. A doubling time of about 4 days was calculated for *Pheocystis poucheti* which numerically dominated the phytoplankton at station 3002 (Fig. 4). The ammonium maximum at 15 m was coincident with a shift in phytoplankton species composition to a community co-dominated by *Biddulphia* sp. and *Prorocentrum* sp. which doubled in about 3.5 days. A doubling time of about 5 days was calculated for the surface sample at station 3038 where *Cylindropyxis tremulens* and *Thalassiosira aestivalis* dominated the phytoplankton. Nitrogen doubling times were similar up to 15 m, below which doubling times ranged from 16 to 30 days. Nitrogen uptake was observed at the 0.1% light depth; however, carbon fixation was usually not different from zero at the 1% light depth. There was a transition from *Pheocystis poucheti* which formed blooms near the surface to a phytoplankton community dominated by diatoms and dinoflagellates as the photic zone deepened with the sinking of the chlorophyll maximum layer in the shelf break front.

The pattern exhibited by chlorophyll and nitrate maps and in hydrocast data suggests that the phytoplankton bloom in the shelf break front of the southeastern Bering Sea was constrained to a narrow ribbon parallel to the 100 fathom isobath. A chlorophyll maximum was always observed along the 170 km length of the front but the maximum was not always at the surface. The chlorophyll maximum persisted in the shelf break front through September 1978 and was observed again during spring 1979.

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## Hypersaline solutions interact with organic detritus to produce oil

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Salt is a major factor in the origin of petroleum. It leads to the extraction of hydrocarbons from sediments and the formation of oil droplets. Also, salt domes and their cap rocks may trap oil<sup>1</sup>. Although the diagenesis of salt and brines is well understood, little is known what salty brines may do to infalling organic detritus. Using results from the Caspian Sea and the North Sea we show here that hypersaline solutions close to the sediment-water interface will cure organic matter and that this 'pickling effect' is essential for obtaining black and oil shales.

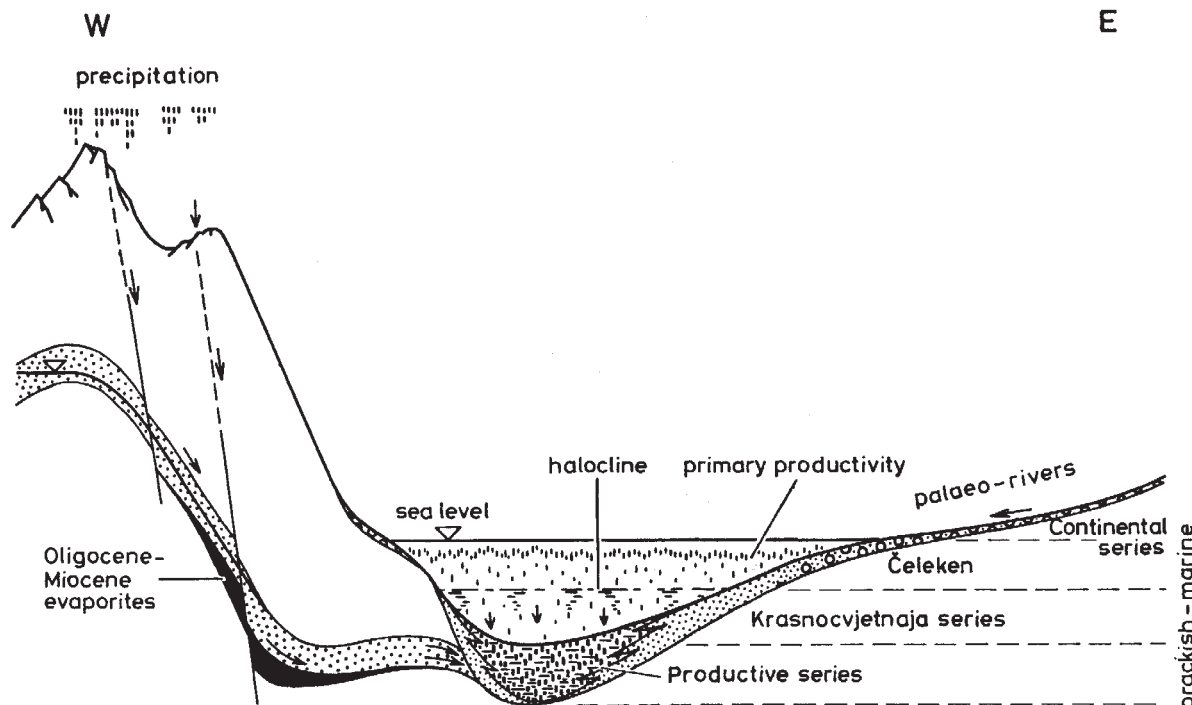


Fig. 1 South-Caspian depression during Kimmer/Akchagyl time; evaporite series are shown in black.

The Black Sea is considered the prototype environment for the black shale facies<sup>2</sup>. During the RV Glomar Challenger DSDP Leg 42B a series of black shales, oil shales and sapropels of Quaternary age were recovered from the Black Sea deep basin at a water depth of about 2,000 m (ref. 3). The 'euxinic events' responsible for this facies pattern are all characterised by saline spills or brine invasions, which in turn led to a midwater halocline and a meromictic basin. Note that oil shale facies develops in a shallow water environment at a water depth of at most a few hundred metres<sup>4</sup>; hypersaline conditions with salinities reaching 100‰ are indicated<sup>5</sup>. In contrast, sapropels are confined to a deep water meromictic sea and salty brines covering the topmost sediment are of lower salinity<sup>4</sup>. Oil shales have ages between 1 and 2 Myr, an organic C content between 0.5 and 2%, and downhole temperature measurements indicate a thermal gradient between 30 and 50 °C per km (ref. 6). These results suggest that hydrocarbon formation in the Black Sea was fast, proceeded at low temperature, and was mediated by syngenetic salt invasions.

In the light of these data we examined two test regions, the Caspian Sea and the North Sea, where we believe similar environmental conditions did prevail except that the geological processes have already led to the accumulation of sizeable oil deposits<sup>7</sup>.

During the Pontic marine phase (lower Pliocene) wide parts of Russia and southeastern Europe were covered by a land-locked shallow sea<sup>8</sup>. The subsequent Kimmer regression reduced the sea to a few isolated basins located in the region of the present south-Caspian depression<sup>9</sup>. The Kimmer climate was arid and the palaeo-rivers Volga, Amudarja and others carried a high salt load to a comparatively small Caspian drainage basin. Pliocene orogeny, still active during the Kimmer, led to the formation of mountains in the vicinity of the Caspian Sea<sup>10</sup>. Groundwater under high pressure within the tilted rock formation moved towards the south-Caspian depression where it was released in the form of Artesian wells. Since the aquifer drained evaporites of Oligocene-Miocene age, mineralisation of the groundwater must have been substantial.

The geological and environmental situation around Kimmer and Akchagyl time is depicted in Fig. 1. Bleeding of groundwater aquifers below sea level combined with brine migration from lagoons resulted in the accumulation of salty brine pools in morphological deeps; a midwater halocline separated the brines from normal Caspian Sea water. The Akchagyl transgression following the Kimmer regression produced a facies succession from clays to sands to gravel (Productive, Krasnocvjtnaja Čeleken, Continental series); brackish-marine and continental deposits mark this event<sup>11</sup>. High primary productivity is indicated, and consequently the Productive series below the halocline received a high input of organic matter and the anoxic conditions established preserved the infalling organic detritus. As the Akchagyl transgression took place at about the Tertiary/Quaternary boundary, generation of hydrocarbons and formation of oil traps must have been quite rapid.

Our second test case, the North Sea, follows an almost identical pattern, except that the black shale event was much earlier, during the Upper Jurassic<sup>12</sup>. A schematic cross-section along a line from Norway to Scotland at the time of the formation of the Kimmeridge clay (Malm) depicts the functionality of the Zechstein and Keuper evaporite series for the generation of a salty groundwater aquifer (Fig. 2). Such a mineralisation and the subsequent release to the basin of deposition was possible at Kimmeridge time because the potential needed for the Artesian pressure was generated at that time by tectonic uplift in Scandinavia and tectonic subsidence in the vicinity of the Central Graben. The water depth in the Central Graben during the Upper Jurassic has been estimated to be at about 1,000 m and a few hundred metres close to the shelf area<sup>13</sup>. In short, the generation of oil shale facies required euxinic conditions and a hypersaline syngenetic environment close to the sediment-water interface. Note that the generation of hydrocarbons could already have started during the Kimmeridgian tectonic phase. The presence of North Sea oil in younger rock formations, for instance, of the early Tertiary, may be linked with hypersaline anoxic events in the Eocene and Oligocene. Alternatively, a



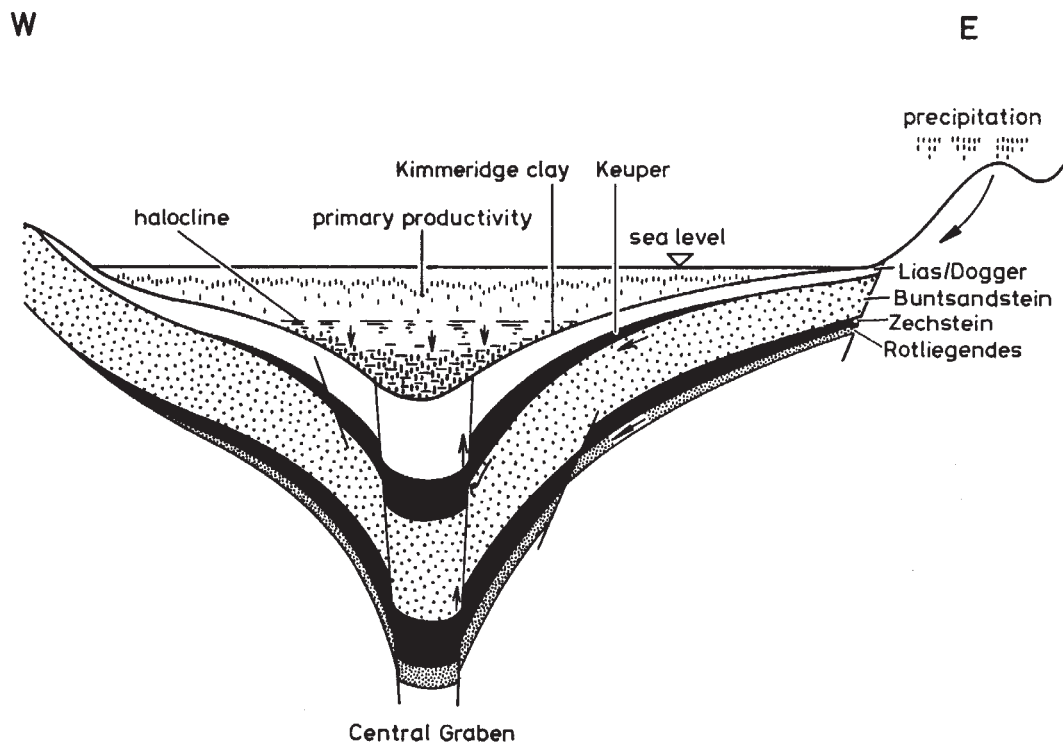


Fig. 2 North Sea at Kimmeridge time; evaporite series are shown in black.

migration of Jurassic petroleum into younger reservoir rocks is conceivable.

Salt water driven by Artesian forces is presently expelled from Red Sea sediments and accumulates in the so-called 'hot brine pools' (ref. 14). The brines are released in pulses which are in phase with pluvial incidents. An identical situation exists in some deep lakes of the East African rift system, most notably in Lake Kivu<sup>15</sup>. In both areas the brines extend from the sea or lake floor up to 200 m into the open water column and are separated from the upper water layer by one or a series of haloclines. Such a decoupling of the upper from the lower water layer will generate anoxic conditions at depth.

Salt invasions will drastically affect marine life in sediment and water. Higher forms of aquatic life become wiped out completely, and microbial population patterns undergo distinct changes in response to, for example, the degree of salinity, ionic composition or brine temperature<sup>16</sup>. Some of these changes are limiting, others are accelerating factors for microbial productivity. High general salinity combined with high calcium content might even act bacteriostatically on sulphate-reducing bacteria<sup>17</sup>. In contrast, halophilic bacteria may thrive in salty waters<sup>18</sup>. The establishment of characteristic microbial food chains or food webs adapted to the new environmental conditions can lead to a transformation of low molecular weight carbon compounds such as CO, CO<sub>2</sub>, CH<sub>4</sub>, organic acids into bacterial biomass<sup>18</sup>. The microbial complexity of brine systems is well documented in the work on Red Sea brine pools<sup>17</sup>, Lake Kivu<sup>15</sup> or the Black Sea<sup>19</sup>.

With respect to oil shale formation the most favourable environment seems to be one in which decomposition of high molecular weight organic matter is kept low and synthesis of bacterial biomass from low molecular weight carbon compounds is high. In such conditions, infalling organic debris of marine and terrestrial origin has a good chance of 'surviving' early diagenesis. This, combined with additional increments from microbiological organic matter, yields the high molecular weight precursor material for the generation of oil.

The formation of oil shales requires high primary productivity, hypersaline conditions at depth, and a gradually subsiding basin. Microorganisms such as methane producers, methane oxidisers (close to the halocline), halophilic bacteria or thermophilic bacteria will supplement the organic input into the sediment. The activity of organisms consuming organic matter, such as sulphate reducers, must be low. The salty waters will sink into the sediment and impregnate the rock formation. Most of the salt in petroleum brine waters has this point of origin. Curing by salt will preserve and condense organic matter. For this reason kerogen in oil shale is of a different nature from kerogen in an ordinary marine or freshwater shale. In taking these environmental and geological factors into consideration, oil exploration may take on some new dimensions.

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# Oldest known eutherian stapes and a marsupial petrosal bone from the Late Cretaceous of North America

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**Cranial remains of Late Cretaceous therian mammals are rarely found in North America and are usually limited to isolated petrosal bones. The oldest known associated stapes and petrosal bone of a Late Cretaceous eutherian are reported here. Comparison with a Late Cretaceous marsupial petrosal bone gives a further indication of the probable condition of several character states of the ancestral mammalian (or at least therian) middle ear, and also indicates the antiquity of various aspects of the middle ear of extant eutherians and marsupials.**

A worn and isolated eutherian petrosal, lacking most of the mastoid process, was identified in the collections of the US National Museum of Natural History. The specimen, USNM 215118 (Fig. 1a, e), comes from the Late Cretaceous Bug Creek Anthills local fauna (Hell Creek Formation, McCone County, Montana)<sup>1</sup>. MacIntyre<sup>2</sup> described several well preserved eutherian petrosals from this local fauna which he called 'ferungulate' and 'unguiculate' types. The former probably belongs to the arctocyonid condylarth, *Protungulatum donnae*, and the latter to the palaeoryctid proteutherian, *Procerberus formicarium*. USNM 215118 is of the latter type and is unique in preserving a broken but complete stapes wedged in the cochlear labyrinth (Fig. 1a, b). The other petrosal described here also comes from the Hell Creek Formation, but about 60 km to the west, in Garfield County, Montana. This well preserved specimen, UCMF 118262, is almost certainly a marsupial, based on its provenience and morphology. It is slightly smaller than the only other known Late Cretaceous marsupial petrosal, that of the stagodontid, *Didelphodon vorax*<sup>3</sup>. Based on size, UCMF

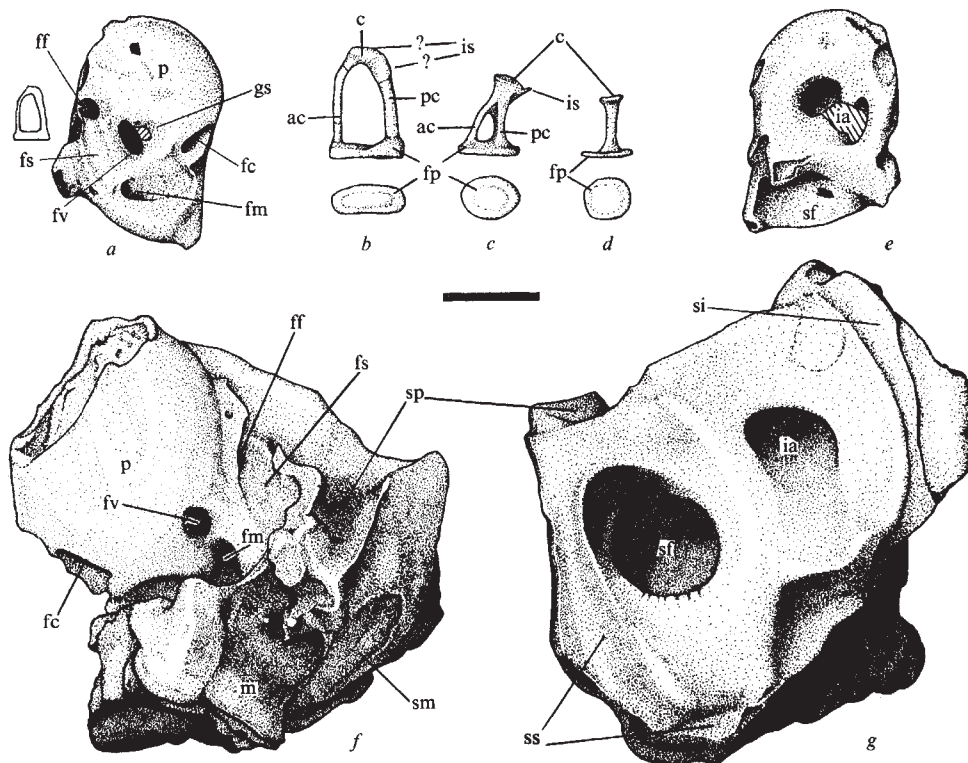
118262 probably belongs either to one of the pedomysids, *Pediomys hatcheri* or *P. florencae*, or to the didelphid, *Alphadon rhaister*.

The stapes associated with the eutherian petrosal has an elongate footplate. The crura are quite gracile and set near either end of the footplate, and the stapedia foramen is large. The stapedia muscle probably inserted on the capitulum or on the thickened portion of the posterior crus immediately below (medial to) the capitulum (is, Fig. 1b). This stapedia morphology is seen in various extant eutherians<sup>4</sup>. Although the Late Cretaceous marsupial petrosal has no stapes, the shape of the footplate can be estimated from the shape of the fenestra vestibuli (fv, Fig. 1f), as can be done for the Late Cretaceous eutherian petrosal (fv, Fig. 1a) because in mammals the fenestra vestibuli outlines the shape of the adjacent stapedia footplate.

Segall<sup>5</sup> quantified the shape of the footplate or fenestra vestibuli as the ratio of length to width. Among didelphid marsupials he found ratios of 1.3–1.5, with the exception of *Dromiciops* at about 2.1. *Dromiciops* has a highly modified ear region and is not considered to be a didelphid by some authors<sup>6</sup>. The fenestra vestibuli of UCMF 118262 has a ratio of about 1.4, the same as for *Didelphodon*. This value lies within the range measured for modern didelphids. In contrast, the eutherians (mostly lipotyphlans) examined by Segall have ratios ranging from 1.8 to 2.9. The ratio in the new eutherian petrosal, USNM 215118, is 2.4 based on the stapedia footplate and 2.1 based on the slightly damaged fenestra vestibuli. The figures of the 'ferungulate' eutherian petrosal of MacIntyre<sup>2</sup> also indicate a ratio of 2.0 or more based on the fenestra vestibuli.

Primitively, mammals (and advanced mammal-like reptiles<sup>7</sup>) had a circular or possibly slightly oval stapedia footplate (equal to a ratio of about 1.0). Thus, the elongate footplates of the new Late Cretaceous eutherian, many extant eutherians and some extant marsupials (such as *Macropus*<sup>5</sup>) represent a derived condition. The smaller ratio in extant didelphid marsupials and in the new Late Cretaceous marsupial petrosal indicates a less elongate stapedia footplate, resembling more closely the circular footplate primitive for mammals.

There is less agreement about the primitive mammalian condition of the crura of the stapes. In monotremes and some marsupials with a circular or slightly oval footplate, the stapes is



**Fig. 1** Right petrosal (a, e) and stapes (a, b) of 'unguiculate' eutherian, USNM 215118, and left petrosal (f, g) of marsupial, UCMF 118262, both from Hell Creek Formation, Montana; right stapes of *Didelphis virginiana* (c) and *Tachyglossus aculeatus* (d); a, f, ventrolateral view, anterior approximately towards top; b, c, d, dorsal and medial views, anterior to left; e, g, dorsomedial view, anterior approximately towards top. ac, anterior crus; c, capitulum; fc, fenestra cochleae; ff, foramen for facial nerve; fm, fossa for origin of stapedius muscle; fp, footplate; fs, sulcus for facial nerve (broken bony canal in marsupial); fv, fenestra vestibuli; gs, groove for stapedia artery; ia, internal acoustic meatus; is, insertion for stapedius muscle; m, mastoid portion of petrosal; p, promontorium of petrosal; pc, posterior crus; sf, subarcuate fossa (broken in eutherian); si, sulcus for inferior petrosal sinus; sm, sulcus for vein leading to mastoid foramen; sp, ? sulcus for portion of transverse sinus leading to postglenoid foramen; ss, posterodorsal portion of sulcus for sigmoid sinus. Scale bar, 2 mm (a, d, e, f, g) and 1 mm (b, c).



columelliform and lacks a stapedia foramen. Segall<sup>5</sup> believes that this is the primitive mammalian condition. Henson<sup>8</sup> follows Goodrich<sup>9</sup> in believing that it is a derived condition.

In addition to many extant mammals, some extant lizards, birds and amphibians<sup>8,9</sup> have a perforate stapes (possess a foramen) that transmits the stapedia artery. In some mammal-like reptiles such as *Thrinaxodon*<sup>7</sup> the stapes had a large stapedia foramen. Whether a stapedia artery traversed this foramen is not known, but it seems likely. The perforate stapes of most mammals and various other fossil and extant tetrapods suggests that this was the primitive mammalian, if not primitive tetrapod condition. Thus, the primitive condition of the crura of the stapes among mammals would resemble the morphology of *Didelphis* (Fig. 1c) more closely than that of a monotreme (Fig. 1d). Both the columelliform crus of the monotreme stapes and probably the stirrup-like crura of the Late Cretaceous eutherian stapes (Fig. 1b) would be derived conditions.

The two new petrosals show several grooves for parts of the arterial and venous systems. Such grooves indicate the patterns of these systems in later fossil and extant eutherians and marsupials. Due to wear, the Late Cretaceous eutherian petrosal has only the shallow, but distinct groove for the stapedia artery (gs, Fig. 1a). Based on better specimens of the same type of petrosal<sup>2</sup>, it almost certainly also had grooves for the medial internal carotid and promontory arteries. In contrast, the well preserved Late Cretaceous marsupial petrosal shows no trace of grooves for any of these arteries.

Both patterns are consistent with what is known of the evolution of the internal carotid circulation in eutherians and marsupials. The common ancestor of eutherians and marsupials had a medial internal carotid that passed medial to the petrosal, leaving little or no trace on this bone, and a stapedia artery that pierced the stapes, possibly leaving some trace on the petrosal<sup>10</sup>. Various lines of evidence suggest that the earliest eutherians maintained this general pattern, adding a neomorphic vessel, the promontory artery, which crossed the lateral side of the promontorium<sup>10</sup>. However, embryological evidence<sup>11</sup> suggests that the eutherian promontory artery represents a shift of the medial internal carotid to the promontorium.

In contrast, the evidence from extant marsupials indicates they neither developed a promontory artery nor shifted an artery to this position<sup>10</sup>. In addition, reports of a branch of the internal carotid passing through a groove or bony canal on the medial side of the marsupial petrosal<sup>12,13</sup> have not been substantiated<sup>14</sup>. Thus, such a groove in the Late Cretaceous marsupial petrosal (si, Fig. 1g) carried a portion of the venous system, the inferior petrosal sinus, not a branch of the internal carotid. Extant marsupials that have been studied usually have the medial internal carotid, while the stapedia artery is reduced<sup>15</sup>; seldom does either leave a trace on the petrosal. *Didelphodon* and the new Late Cretaceous marsupial petrosal similarly lack grooves for the medial internal carotid and stapedia arteries. By analogy, if not homology, both Late Cretaceous marsupials probably had a medial internal carotid, but lacked or had a greatly reduced stapedia artery.

These comparisons indicate the antiquity of several characters of the middle ear seen in various extant eutherians and marsupials. By the Late Cretaceous the stapes had been modified in at least some eutherians. Functional implications remain speculative. At the simplest mechanical level, the torque about the long axis of an elongate stapedia footplate such as in the Late Cretaceous stapes is less than about the axis of a circular footplate of the same area. Thus, even such a minor modification as the lengthening of the footplate may have enhanced the hearing capabilities of this eutherian. The much less elongate footplate (indicated by the shape of the fenestra vestibuli) of Late Cretaceous marsupials is a retention of a more primitive condition that is maintained in extant didelphids. The elongate footplate in various other extant marsupials evolved in parallel with the eutherians.

In contrast to stapedia morphology, the portions of the internal carotid system that cross the middle ear show a pattern

of mosaic evolution in Late Cretaceous eutherians and marsupials. The common ancestor of mammals almost certainly possessed a medial internal carotid and a stapedia artery. By the Late Cretaceous, marsupials had greatly reduced or lost the latter vessel, whereas contemporaneous eutherians maintained it. Also, if the interpretations of the various grooves are correct, eutherians had developed a neomorphic vessel, the promontory artery, by the Late Cretaceous. Again, functional implications are speculative, but these various patterns may simply reflect a shifting of the arterial blood supply to portions of the brain and orbit; or in the case of eutherians, the probable appearance of a new artery may have served as a sort of primitive rete mirabile for cooling blood destined for the brain.

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## Effective mutualism between sequentially flowering plant species

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Since the theoretical paper of Levin and Anderson<sup>1</sup>, it has been widely recognised that animal pollinators represent resources for which plants can compete. Competition for pollination might take several forms and act as a powerful selective force in establishing or maintaining sequential flowering among sympatric species<sup>2</sup>. Sequential flowering in Arctic<sup>3</sup>, temperate<sup>4-9</sup> and neotropical<sup>10-13</sup> plant assemblages has been interpreted as an evolutionary result of competition for pollination. This interpretation may prove correct in many cases, although strong evidence for competition is available for few systems<sup>9,14</sup>. Heinrich and Raven<sup>15</sup> (see also Baker<sup>16</sup> and Baker *et al.*<sup>17</sup>) pointed out that sympatric plant species may act as mutualistic partners at the same time that their sequential flowering is maintained by competition. We develop this hypothesis here explicitly and present evidence that effective mutualism occurs between two species which also compete for pollination.

To illustrate the hypothesis of mutualism we consider three sequentially flowering species which share pollinators (Fig. 1). If

**Table 1** Densities of flowers, seed sets and census counts of hummingbirds from the RMBL

| Year | Peak density of <i>D. nelsonii</i> flowers* | Peak density of <i>I. aggregata</i> flowers* | <i>I. aggregata</i> seed set† | Overall broad-tail census count‡ | Late broad-tail census count§ | Overall rufous census count¶ |
|------|---------------------------------------------|----------------------------------------------|-------------------------------|----------------------------------|-------------------------------|------------------------------|
| 1975 | 25.0±9.20 (6)                               | 29.3±13.26 (6)                               | 10.1±0.73 (37)                | 10.2±1.44 (14)                   | 8.6±2.09 (7)                  | 7.1±2.79 (8)                 |
| 1976 | 30.8±9.41 (6)                               | 27.8±9.32 (6)                                | 12.4±0.68 (47)                | —                                | —                             | —                            |
| 1977 | 0.8±0.54 (6)                                | 18.7±7.70 (6)                                | 7.6±0.46 (59)                 | 8.0±1.13 (11)                    | 6.9±1.45 (7)                  | 7.2±6.95 (5)                 |
| 1978 | 7.2±2.36 (6)                                | 39.3±13.99 (6)                               | 7.1±0.71 (43)                 | 5.0±1.86 (10)                    | 5.0±1.86 (10)                 | 6.9±2.43 (8)                 |

\* Values are mean number of flowers per 4 m<sup>2</sup> plot ±1 s.e.m. with sample sizes (no. of plots) in parentheses. Both species were counted every 2–3 days in 6 permanent census plots. The 1977 *D. nelsonii* value is a slight underestimate as the first census was taken when density of this species was already declining.

† Values are mean seed sets per flower ±1 s.e.m., with sample sizes (no. of flowers) in parentheses. Flowers were taken from unmanipulated plants in the meadow containing flower census plots.

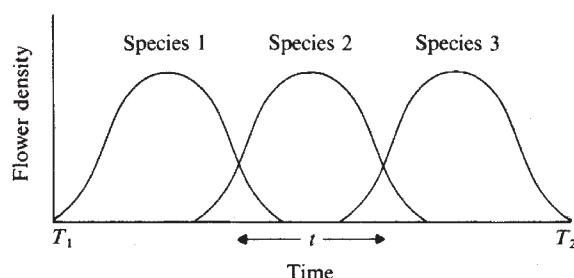
‡ Values are mean number of flights by male broad-tailed hummingbirds per 10 min census ±1 s.e.m., with sample sizes (no. of censuses) in parentheses. Flights were censused at a standard time of day in the meadow containing flower census plots; no censuses were conducted in 1976. Censuses cover the flowering season of both species in 1975 and 1977 and flowering of *I. aggregata* alone in 1978.

§ Values are given as before. Only censuses conducted during flowering of *I. aggregata* have been included for each year.

¶ Values are given as before, except for male rufous rather than broad-tailed hummingbirds. Censuses cover the flowering season of *I. aggregata* only, as rufous hummingbirds were not present during flowering of *D. nelsonii*.

the time between peaks of flowering of species 1 and 3 is sufficient for the absence of species 2 to cause appreciable mortality, emigration, or loss of fecundity to pollinators, then species 2 increases immediate reproductive potential of species 3 by maintaining pollinators through some time interval  $t$ . When the flowering season (the period  $T_1 \rightarrow T_2$ ) covers an entire year all three species are engaged in mutualism. When  $T_1 \rightarrow T_2$  represents part of a year, a similar interaction occurs if colonisation, survival, or fecundity of pollinators depend on overall abundance of flowers or on their continuous availability throughout some critical period.

For this mutualism to occur numbers of pollinators must be reduced locally in response to reduced numbers of flowers. This seems especially likely for obligate flower visitors such as



**Fig. 1** Idealised sequential flowering of three sympatric species sharing common pollinators. Removal of any species leaves a period  $t$  during which few or no flowers are available locally.

bees, bombyllid and syrphid flies, hawkmoths and most butterflies<sup>18–20</sup>. For example, survival and reproduction of bumblebee colonies seem to reflect availability of flowers<sup>21</sup>. Pollinators which are not obligate flower visitors also may react strongly to reduced flowering. For example, densities of territorial hummingbirds and sunbirds reflect densities of flowers<sup>22–25</sup> and poor flowering is associated with reduced survival and nesting success of hummingbirds<sup>25–27</sup>.

Our previous work<sup>9</sup> suggests that competition for hummingbird pollination maintains sequential flowering of two herbaceous wildflowers, *Delphinium nelsonii* Greene and *Ipomopsis aggregata* (Pursh) V. Grant, around the Rocky Mountain Biological Laboratory (RMBL) in Colorado. From measurements of seed sets and densities of flowers over four summers we have evidence that these two species are also engaged in a mutualism of the kind we describe. Peak densities of *D. nelsonii* flowers were similar in 1975 and 1976 but declined drastically in 1977 and 1978 (Fig. 2, Table 1), presumably due to the 1977 winter drought. Densities of *I. aggregata*

flowers remained relatively constant between years and were uncorrelated with densities of *D. nelsonii* flowers (Table 2a). The mutualism hypothesis predicts that reproductive success of *I. aggregata* will decline in the 2 years of low density of *D. nelsonii*. This prediction is supported by reduced seed sets of *I. aggregata* in 1977 and 1978 (Table 1). The slope of the linear regression of seed sets on peak densities of *D. nelsonii* flowers over all years is positive, and the correlation between these variables is significant (Table 2b).

It could be argued that reduced fecundity of *I. aggregata* in 1977 and 1978 was also a subtle effect of drought rather than of reduced availability of hummingbird pollinators after failure of *D. nelsonii* flowering. However, census records suggest that activity of male broad-tailed hummingbirds (*Selasphorus platycercus* Swainson) was reduced in 1977 and 1978 relative to 1975 as predicted by the mutualism hypothesis. These birds, along with rufous hummingbirds (*S. rufus* Gmelin), are the major pollinators of *I. aggregata* around the RMBL<sup>9</sup>, and this species is self-incompatible (1978 seed sets of self-pollinated flowers on five bagged plants compared with those of open-pollinated flowers on eight unbagged plants were  $0.3 \pm 0.08$  ( $n = 256$ ) compared with  $7.1 \pm 0.71$  ( $n = 43$ ). However, rufous hummingbirds are migratory transients who usually arrive after flowering of *D. nelsonii* and whose activity did not fluctuate substantially

**Table 2** Linear regressions and correlations between seed sets, densities of flowers and census counts of hummingbirds

a Peak densities of *I. aggregata* flowers against peak densities of *D. nelsonii* flowers

$$I. aggregata = 0.08 (D. nelsonii) + 27.44$$

$$r = 0.14 \quad d.f. = 2 \quad P > 0.5 \text{ NS}$$

b Raw *I. aggregata* seed sets against peak densities of *D. nelsonii* flowers

$$\text{seed set} = 0.15 (D. nelsonii) + 7.04$$

$$r = 0.41 \quad d.f. = 183 \quad P < 0.001$$

c Raw *I. aggregata* seed sets against overall census counts of broad-tailed hummingbirds

$$\text{seed set} = 0.58 (\text{census count}) + 3.65$$

$$r = 0.27 \quad d.f. = 136 \quad P < 0.01$$

d Raw *I. aggregata* seed sets against late census counts of broad-tailed hummingbirds

$$\text{seed set} = 0.78 (\text{census count}) + 2.90$$

$$r = 0.24 \quad d.f. = 136 \quad P < 0.01$$

All regressions were run using mean values from Table 1, except that *I. aggregata* seed sets are original raw values rather than means for each year. Correlation coefficients and significance values are shown for the association between each set of variables. NS, not significant.



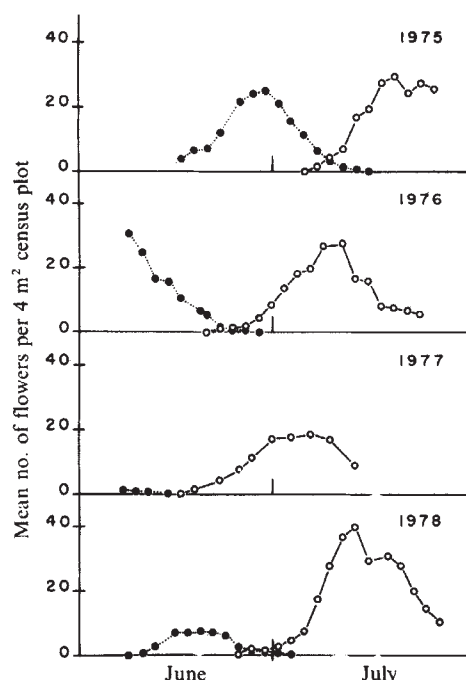


Fig. 2 Sequential flowering of *D. nelsonii* (●) and *I. aggregata* (○) at the RMBL during four consecutive summers. Note the poor flowering of *D. nelsonii* in 1977 and 1978.

between years (Table 1). In contrast, broad-tailed hummingbirds are breeding residents who colonise the RMBL area largely in response to flowering of *D. nelsonii*<sup>27</sup>. Slopes of linear regressions of *I. aggregata* seed sets on yearly mean census counts of broad-tailed hummingbirds are positive, and correlations between these variables are significant (Table 2c, d).

In contrast to competition for pollination, it is difficult to see how the mutualism discussed here could establish or maintain sequential flowering through selection acting on individual plants or even on alternate plant assemblages. We consider effective mutualism to be an unanticipated outcome, rather than a cause, of divergence in flowering times of sympatric species.

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## 'Time-intensity trading' in locust auditory interneurons

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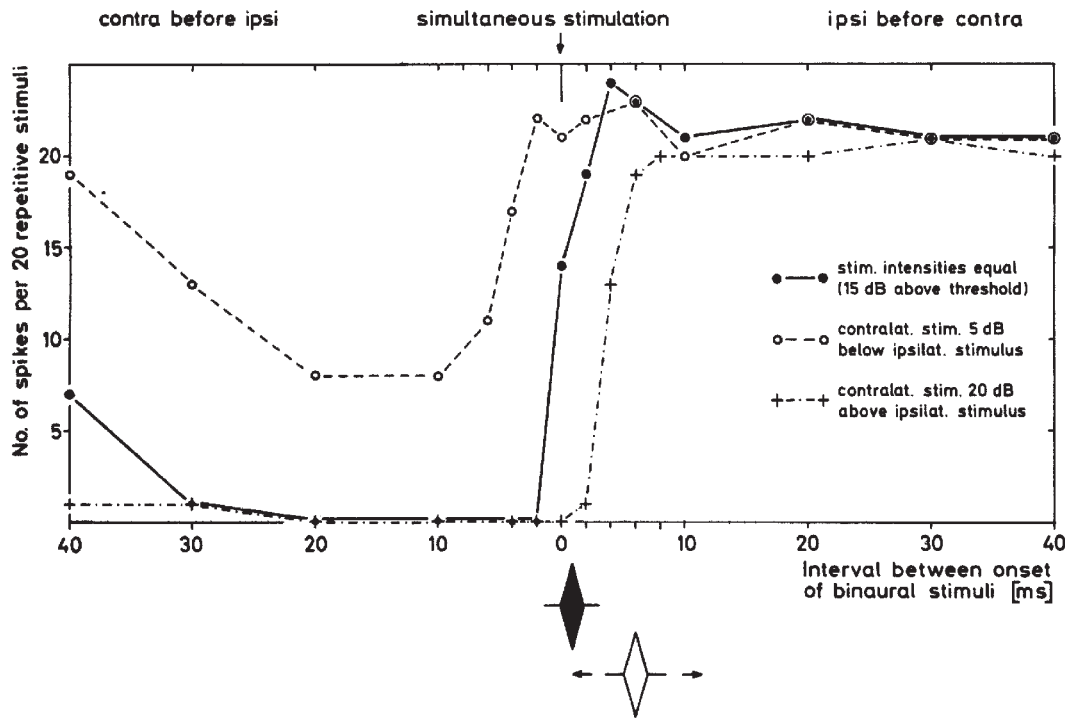
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In the locust the firing rate of tympanal nerve fibres encodes the direction of the sound source, and this has been used as fundamental evidence for the directional sensitivity of each of the tympanal organs<sup>1–4</sup>. As there is a minute disparity between the two organs (in *Locusta* this distance is about 4 mm), it was generally accepted that the physical time cue—maximally about 10  $\mu$ s—cannot be used for sound localisation. However, Mörchén *et al.* have found that in single receptor fibres of the locust, the magnitude of excitation is highly correlated with the latency of nervous activity<sup>5</sup>. In their study a sound source was moved around the animal and the latency of a neural discharge increased by up to 6 ms as the firing rate decreased. This 'neural time cue' exceeded the physical one by a factor of 100–1,000. Comparison of the inputs from the two organs shows that there are two neural codes available (spike count and latency), each of which can adequately specify the direction of the sound source. We report here that the subsequent synapses process this dual mode of directional coding and that both the rate of binaural spike discharges and latency differences influence the activity of auditory interneurons.

We recorded the responses of a pair of identified acoustic interneurons in *Locusta migratoria* from the connectives between the meso- and metathoracic ganglia. This pair of auditory units (type B<sub>1</sub>)<sup>6</sup> is excited ipsilaterally and inhibited from the contralateral side, and thus it is appropriate for the study of binaural processing. To provide an effective uncoupling of the two organs the tympanal membranes were displaced by contact-mechanical stimulation. Two custom-made piezoelectric transducers (similar to those described elsewhere<sup>7</sup>) were used and driven with a sinusoidal signal of 69 kHz; such a frequency eliminates conventional acoustic stimulation of auditory receptor cells (compare ref. 8) but nevertheless activates them adequately (for control experiments see below). With this method the 'crosstalk barrier' between both ears was greater than 55 dB. In each experiment the activities from the units on both sides of the central nervous system (CNS) were recorded successively to calibrate the stimulus intensities of the two probes (for control experiments see below).

The effects of interaural time and intensity differences on the response of the auditory interneurone are shown in Fig. 1. The solid line represents equal stimulus intensities at both ears. By stimulating the ipsilateral (excitatory) ear alone, a maximal response was achieved; this corresponded to the binaural stimulus situation with the ipsilateral stimulus preceding the contralateral (inhibitory) stimulus in a range from 4 to 40 ms. When the inhibitory stimulus preceded the excitatory one the activation of this unit was completely suppressed (note that the steep slope of the curve occurred in a time window of  $\sim \pm 2$  ms for either the ipsi- or contralateral stimulus leading). Thus the synaptic inputs of this interneurone are highly sensitive to time differences of the order of a few milliseconds. It was necessary for the inhibitory stimulus to precede the excitatory one by more than 80 ms for it to be completely ineffective (not shown in Fig. 1, for comparison see Fig. 2).

A decrease in the contralateral (inhibitory) stimulus of only 5 dB shifted the steep slope of the curve in Fig. 1 to the left (dotted line) where the midpoint is defined by simultaneous stimuli. This means that inhibitory effects were only effective when preceding excitation by more than 2 ms, but not suppressing the activity completely. When the contralateral stimulus was reduced by a further 5 dB, it had no effect on the response of the interneurone. However, when the intensity of the contralateral



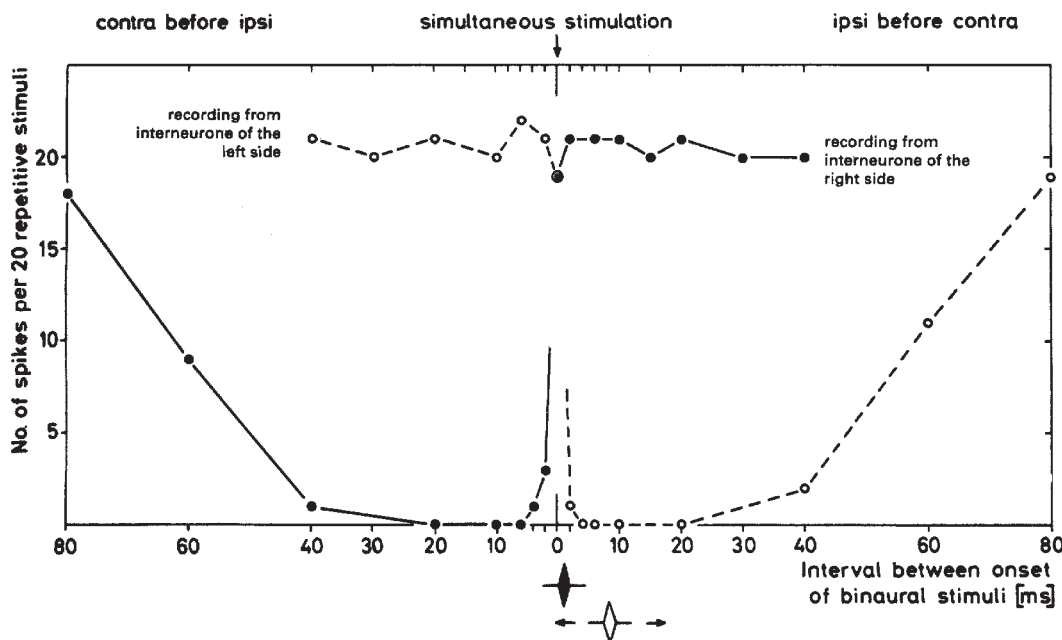
**Fig. 1** Effects of interaural time and intensity differences on the response of an auditory interneurone in the locust to short contact-mechanical stimuli on the tympanic membranes; activation by ipsilateral ( $\blacklozenge$ ), inhibition by contralateral ( $\diamond$ ) stimulus (stimulus frequency 69 kHz, rise- and decay-time 1 ms, plateau 0 ms, repetition rate 1 per s), for further indications see other figure legends. Note that the steepest slope of the curves produced by various interaural time differences can be traded by changing the interaural intensities.

stimulus was increased relative to the ipsilateral one the steep slope of the curve shifted to the right (shown in Fig. 1 for 20 dB difference, dashed line). This means that with more intense contralateral stimuli the inhibition was effective even when it followed the ipsilateral stimulus by  $\sim 4$  ms. (A further increase of the contralateral stimulus intensity had no additional effect on this curve.)

The data presented in Fig. 1 reveal that in the CNS of insects there is a phenomenon similar to the time-intensity trading well known for vertebrates (reviewed by Erulkar<sup>9</sup>). But in insects the 'time cue' is based on the physiological properties of scolopidian receptor cells and has another order of magnitude. Furthermore, the data show that within a given 'time and intensity window' such interneurons are highly sensitive to binaural

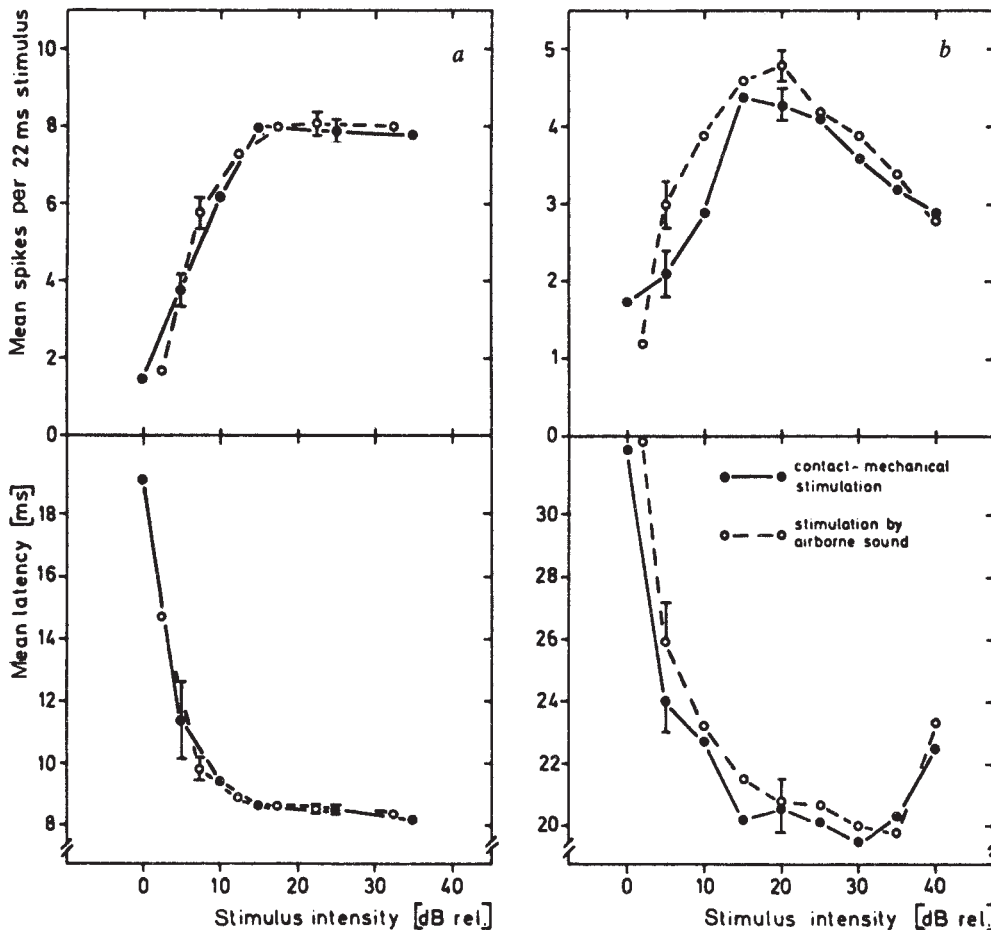
changes. Specifically interaural time differences of the order of 4 to 6 ms and interaural intensity differences of about 20 dB will change the activity from maximal excitation to total inhibition.

The experiments with contact-mechanical vibration may present some technical problems (for more details see ref. 10). In particular, with bilateral processing, the question of whether there is symmetrical displacement of the two membranes, and the equivalence of acoustic and contact-mechanical stimulations have to be tested. To answer the first question a control measurement was performed in each experiment and the activities from equivalent units on each side of the CNS were recorded in succession. This procedure was necessary to define the neural thresholds of each unit to the vibratory stimulus, providing controlled stimulus intensities at both ears. In some cases (such



**Fig. 2** Effects of interaural time difference on the response of a pair of auditory interneurons on either side of the CNS in the locust to short contact-mechanical stimuli on the tympanic membranes; activation by ipsilateral ( $\blacklozenge$ ), inhibition by contralateral ( $\diamond$ ) stimulus, 15 dB above threshold respectively (further stimulus configurations see Fig. 1 legend). In each recording the 'leading' trigger and recording side have been reversed. Note the symmetry and the steepest slope of the curves near simultaneous stimulus onsets.





**Fig. 3** Intensity and latency characteristics of a receptor cell (*a*) and an interneurone (*b*) of the locust for contact-mechanical stimulation on the tympanic membrane (vibrating frequency of 69 kHz) and stimulation by airborne sound at best frequencies (for *a*, 16 kHz; for *b*, 10 kHz); for both types of stimuli rise- and decay-time 1 ms, plateau 20 ms, repetition rate 1 per s. Each data point represents 10 stimuli. The abscissa gives the stimulus intensity referred to relative voltage at the vibrating probe (for *a*,  $0 \text{ dB} \pm 42 \text{ mV}_{\text{pp}}$ ; for *b*,  $0 \text{ dB} \pm 21 \text{ mV}_{\text{pp}}$ ) and sound pressure levels in the free-field (for *a*,  $0 \text{ dB} \pm 47 \text{ dB SPL}$ ; for *b*,  $0 \text{ dB} \pm 27 \text{ dB SPL}$ ). The displacement of the tip of the probe (movement forwards and backwards) is about  $2,200 \text{ \AA}$  for  $3 \text{ V}_{\text{pp}}$  input voltage and linear over the whole operating range (calibrated by laser vibrometry). This magnitude of displacement corresponds to physiological values as measured on the cricket ear<sup>12</sup>. The tip of the probe was perpendicularly placed at a point on the membrane, about  $200 \text{ }\mu\text{m}$  ventromedial to a dark cuticular landmark (this position was maintained in all experiments). When the probe was not in contact with the membrane, even the highest voltage used elicited no response; this indicates that only contact-mechanical and no inadvertent acoustic stimuli were effective.

as the experiments presented in Fig. 1) observations were repeated for the corresponding unit on the other side of the CNS, maintaining both stimuli at 15 dB above threshold. In Fig. 2 the corresponding curves are shown for recordings from the right (solid line) and left (dashed line) interneurons. Since the two curves are nearly identical over the whole time axis, this example implies a symmetrical stimulus situation at both ears.

To deal with the second problem, we measured the response characteristics of several receptor cells and some interneurons, each stimulated with the piezotransducer and, after its removal, by airborne sound. As shown by the example in Fig. 3*a*, the receptor cell exhibited identical intensity and latency characteristics to the two stimuli (for similar results in moths see ref. 11). These data strongly suggest that the magnitude of neural excitation at the receptor can be finely controlled by both types of stimulus, which is important for the interpretation of Fig. 1 (see above). The coincidence between the two types of curve was found in both low- and high-frequency receptors. In Fig. 3*b* the corresponding curves for an auditory interneurone are shown. Although this interneurone exhibits a rather complicated intensity characteristic, both pairs of curves are similar. It is evident that at both neuronal levels, in the given circumstances, the contact-mechanical stimulation can be made equivalent to the acoustic free-field situation.

Referring to Fig. 1 we emphasise that by the contact-mechanical stimulation technique the binaural spike code can be separated from the binaural latency difference. The former code could be controlled by stimulus intensity (see Fig. 3), and the latter one was manipulated by experimental time delays between the two stimuli. Clearly both kinds of neuronal code control the activity of the interneurone. This is also true in

free-field conditions, because with ipsilateral sound, the excitation of the ipsilateral receptors precedes inhibition from the contralateral side, which is also smaller in magnitude. In contralateral stimulus conditions this situation changes drastically, as now the greater inhibition precedes reduced excitation. Thus both cues—intensity and the time of arrival of the neural response in the CNS—have important, mutually reinforcing effects on synaptic transmission and ensure the optimal use of the peripherally processed directional information.

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## Ionic basis of the receptor potential in a vertebrate hair cell

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Vertebrate hair cells, the primary receptors of auditory, vestibular and lateral-line organs, occur in epithelia which separate fluids of differing ionic composition. The apical surfaces of hair cells, on which the mechanosensitive hair bundles are situated, face a high- $K^+$  fluid (termed endolymph in the inner ear); the basolateral surfaces instead contact fluid (perilymph or a related substance) of a composition similar to that of other extracellular fluids<sup>1-3</sup>. The universal occurrence of high- $K^+$  fluid on the apical surfaces of hair cells in vertebrates has been taken as evidence that it is important for the transduction process, in particular that it relates to the ionic specificity<sup>4</sup> of the conductance change<sup>5</sup> underlying the receptor potential. There is, however, conflicting experimental evidence regarding this specificity.  $K^+$  has generally been thought to carry the receptor current, as replacement of endolymph with perilymph in the guinea pig cochlea abolishes the extracellularly recorded microphonic potential<sup>6</sup>. Yet microphonic potentials, as well as intracellular receptor potentials, have been recorded in other preparations when the apical surfaces of the hair cells faced instead a high- $Na^+$  saline, and thus when the electrochemical gradient for  $K^+$  was near zero<sup>5,7</sup>.  $Ca^{2+}$  has also been proposed to carry the receptor current<sup>8</sup>, but its concentration is quite low in endolymph<sup>3</sup>, particularly that of the mammalian cochlea<sup>9</sup>. We present evidence here that the receptor current in a vertebrate hair cell is carried *in vivo* by  $K^+$ , but that the transduction channel is in fact nonspecific, being permeable to  $Li^+$ ,  $Na^+$ ,  $K^+$ ,  $Rb^+$ ,  $Cs^+$ ,  $Ca^{2+}$ , and at least one small organic cation.

Hair cells were prepared for intracellular recording as described previously<sup>5,10</sup>. Sacculi were removed from adult bullfrogs and their otolithic membranes peeled away after loosening by mild proteolysis (incubation for 60 min with  $0.03 \text{ mg ml}^{-1}$  subtilopectidase A, EC 3.4.4.16, at  $22^\circ\text{C}$ ). The tissue was positioned in an experimental chamber with standard saline on all cell surfaces and viewed with Nomarski differential interference contrast optics. Individual hair cells were penetrated by two glass microelectrodes, each 100–150 M $\Omega$  in resistance, connected to a simple voltage clamp circuit. To stimulate each impaled cell, its hair bundle was deflected with a fine glass probe ( $0.2 \mu\text{m}$  tip diameter) inserted horizontally between the kinocilium and the rest of the hair bundle. Probes were moved by an electronically controlled, two-dimensional stimulator, over a distance of 1–2  $\mu\text{m}$ , with a 10-Hz, triangle waveform.

We first measured the current-voltage relationship for unstimulated hair cells (Fig. 1). The input resistance measured with two electrodes was 200–300 M $\Omega$  at a holding potential of  $-60 \text{ mV}$ . Because the resistance was higher (200–900 M $\Omega$ ) if measured with one electrode and an active bridge circuit, much of the apparent membrane conductance at this potential resulted from penetration damage. At membrane potentials more positive than about  $-50 \text{ mV}$ , however, the resistance dropped to 6–7 M $\Omega$ . This conductance increase was largely blocked by 1 mM 3,4-diaminopyridine<sup>11</sup>, and is evidently a voltage-dependent  $K^+$  conductance. Such outward or delayed rectification may explain the rather small receptor potentials seen in some hair cell preparations<sup>12,13</sup>: because of the change in slope conductance, a receptor current which generates a receptor potential of 20 mV in bullfrog hair cells at a resting potential of  $-60 \text{ mV}$  produces only 0.5 mV at  $-40 \text{ mV}$ . Figure 1 shows an additional conductance increase, an inward or anomalous rectification, at potentials below  $-100 \text{ mV}$ . Such a rectification

has been hypothesised to explain certain aspects of electrical behaviour of the mammalian cochlea<sup>14</sup>, but has not previously been demonstrated for vertebrate hair cells.

The stimulus-associated current, or receptor current, was measured by presenting periodic mechanical stimuli of saturating amplitude while clamping to various potentials. The receptor current at the holding potential of  $-60 \text{ mV}$  ranged from 45 to 205 pA with a mean of 98 pA ( $\pm 37 \text{ pA}$ , s.d.,  $n = 28$  cells) and with the larger values more representative of healthy cells. Variation in the measured current was caused both by decline in the viability of a cell during prolonged recording and by mechanical drift of the stimulus probe with respect to the responsive range of the hair bundle. The voltage dependence of the receptor current was consequently determined by comparing the receptor current at a test potential to the current at the holding potential immediately before and after the potential step. The receptor current at  $-60 \text{ mV}$  was normalised to 100 pA for each cell, and the receptor current at each test potential then scaled by the same amount. An implicit assumption in this analysis is that the mechanical sensitivity is not also voltage-sensitive, for instance that the responsive range of the hair bundle does not shift as the cell is depolarised.

A representative series of clamp records is shown in Fig. 2a. The receptor current reverses its direction from inward to outward at a membrane potential between  $-9$  and  $+11 \text{ mV}$ . Figure 2b combines normalised data from 24 cells; a linear regression fit to these data indicates a reversal potential of  $-2 \text{ mV}$ . This is not the equilibrium potential for  $Na^+$ ,  $K^+$ , or  $Ca^{2+}$ , since a standard, 124-mM  $Na^+$  saline solution<sup>5</sup> faced all cell surfaces during these experiments. It could, however, result from a stimulus-dependent conductance that is nonspecific—that has a limited ability to discriminate among cations.

We consequently tested several cations using an *in vitro* microphonic technique<sup>15</sup>. The macular epithelium of the sacculus was positioned across a hole between two chambers, so as to separate them ionically and electrically. The potential across

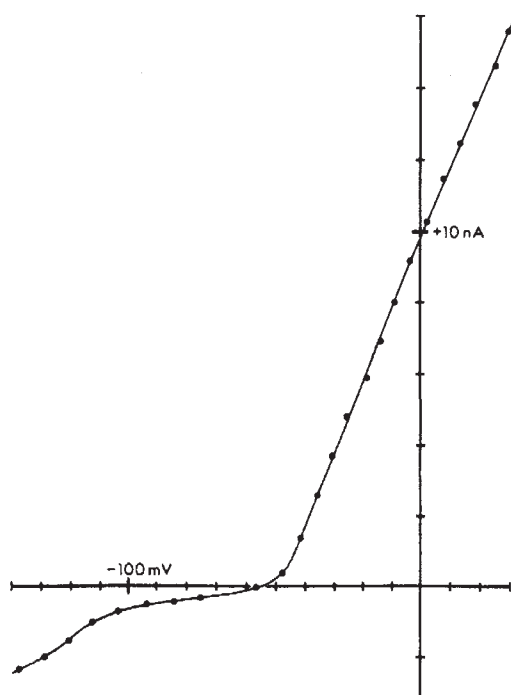
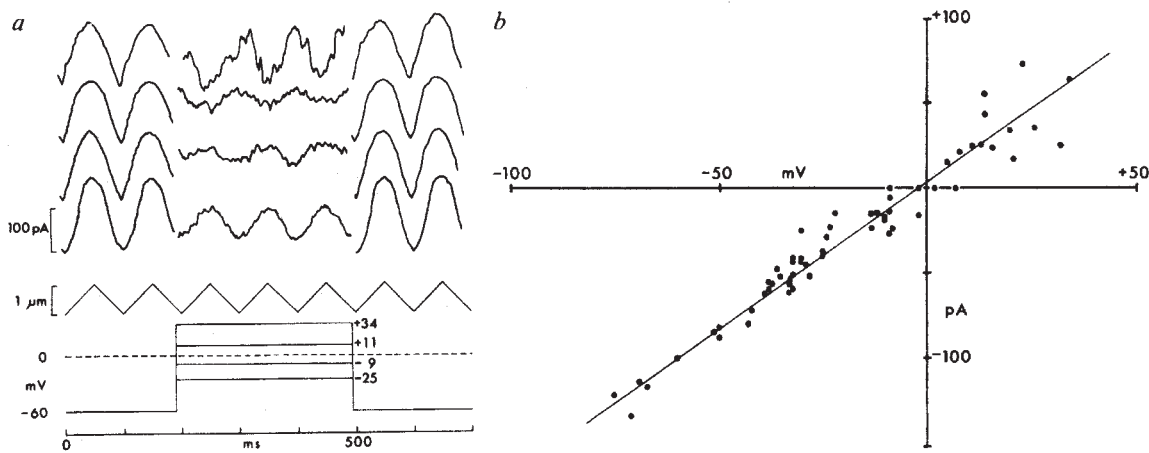


Fig. 1 Current-voltage relationship for a saccular hair cell, measured 50 ms after the start of a voltage step. Inward current is shown as negative. The holding potential was  $-60 \text{ mV}$ . The striking outward rectification which occurs above  $-50 \text{ mV}$  was blocked by 3,4-diaminopyridine and probably corresponds to the delayed rectification of most neurones. Inward or anomalous rectification is evident at potentials below  $-100 \text{ mV}$ .





**Fig. 2** *a*, Receptor currents at a holding potential of  $-60$  mV and at test potentials of  $-25$ ,  $-9$ ,  $+11$ , and  $+34$  mV; inward current is shown as negative. The membrane potential was held at  $-60$  mV for 200 ms, clamped to the test potential for 300 ms, and returned to the holding potential. Each trace represents the average of 10 test steps. Ionic currents that were not stimulus-dependent were electronically subtracted before measuring the receptor current. The hair bundle was continuously moved with a  $1.1$ - $\mu\text{m}$ ,  $10$ -Hz, triangle-wave stimulus, shown below the current records; downward represents a deflection toward the kinocilium. *b*, Magnitude of the receptor current as a function of membrane potential; normalised data from 24 cells. The line is a linear regression fit to the data ( $r^2 = 0.97$ ); its zero-current intercept, the reversal potential, is  $-2$  mV. The external fluid was standard, high- $\text{Na}^+$  saline, so that the reversal potential is not the equilibrium potential of  $\text{Na}^+$ ,  $\text{K}^+$ , or  $\text{Ca}^{2+}$ .

the epithelium was monitored with an electrode in each chamber; a second pair of electrodes was used to pass current to clamp the transepithelial potential at zero. Measuring the transepithelial current, rather than the transepithelial potential, avoided variations in response amplitude caused by slow changes in epithelial resistance. Hair cells were stimulated *en masse* by moving the otolithic membrane, which remained attached to the hair bundles, with a glass probe driven by a piezoelectric element. The stimulus was a saturating step displacement of amplitude  $1.5$   $\mu\text{m}$  and duration  $1$  ms. The otolithic membrane can follow this fast stimulus, and the microphonic current turns on and off in much less than this time<sup>15</sup>. The charging time of the hair cell membrane is much longer, however, so that the intracellular potential should change only slightly during the pulse. The measured current is accordingly dependent on the resting potential and the permeability to the test ion, but not on voltage-sensitive conductances that are secondary to the receptor potential.

The chambers were separately perfused with a test solution on the apical side ( $130$  mM of the relevant monovalent cation,  $0.25$  mM  $\text{Ca}^{2+}$ ,  $130$  mM  $\text{Cl}^-$ ,  $3$  mM D-glucose,  $1$  mM HEPES) and an artificial perilymph on the basolateral side ( $3.63$  mM  $\text{K}^+$ ,  $122$  mM  $\text{Na}^+$ ,  $1.36$  mM  $\text{Ca}^{2+}$ ,  $0.68$  mM  $\text{Mg}^{2+}$ ,  $130$  mM  $\text{Cl}^-$ ,  $3$  mM D-glucose,  $1$  mM HEPES). The apical surfaces of hair cells and supporting cells are bounded by tight junctions, which limit the exchange of ions between the two chambers.

All of the alkali cations supported the microphonic current. The mean relative signal amplitudes from eight experiments were:  $\text{Li}^+$ ,  $0.9$ ;  $\text{Na}^+$ ,  $0.9$ ;  $\text{K}^+$ ,  $1.0$ ;  $\text{Rb}^+$ ,  $1.0$ ; and  $\text{Cs}^+$ ,  $1.0$ . Ammonium ion ( $\text{NH}_4^+$ ) was more permeant, with a relative amplitude of  $1.3$ . To establish whether still larger ionic species could traverse the transduction channel, we bathed the apical surface with test solution containing  $130$  mM tetramethylammonium ion (TMA). This reduced the microphonic current to  $0.2$  of its value for  $130$  mM  $\text{K}^+$ , suggesting that TMA can pass through the transduction channel, although less readily than the alkali cations. To confirm this observation, TMA was tested on single cells using the intracellular voltage clamp method. The reversal potential, with  $130$  mM TMA on all cell surfaces, was  $-25$  mV. Assuming that the extracellular TMA activity is roughly the same as the intracellular  $\text{K}^+$  activity, this indicates a channel permeability to TMA about  $0.4$  that of  $\text{K}^+$  (refs 16, 17). The fact that the permeabilities estimated by ionic current and by reversal potential are not equal suggests that TMA partially

blocks the transduction channel<sup>18</sup>.

The ability of one divalent ion,  $\text{Ca}^{2+}$ , to pass through the transduction channel was also demonstrated by extracellular recording. With  $87$  mM  $\text{CaCl}_2$  in the test solution at the apical surface of the hair cell, the microphonic current was  $0.3$  that produced by  $130$  mM  $\text{K}^+$ . Because its concentration in endolymph is very low,  $\text{Ca}^{2+}$  can carry very little of the receptor current *in vivo*.  $\text{Ca}^{2+}$  does seem, however, to be a necessary cofactor for the response<sup>5,8</sup>: with a  $130$ -mM  $\text{K}^+$  saline, the microphonic current was abolished if the  $\text{Ca}^{2+}$  concentration was reduced below about  $10$   $\mu\text{M}$ .  $\text{Sr}^{2+}$  replaces  $\text{Ca}^{2+}$  in this role, but  $\text{Mg}^{2+}$  and  $\text{Ba}^{2+}$  do not.

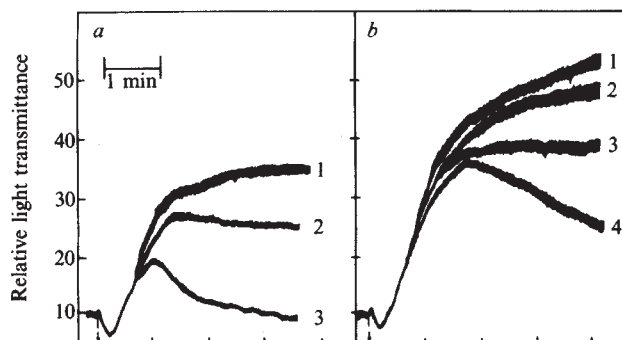
Because the transduction channel in frog saccular hair cells is nonspecific, the transduction current *in vivo* will be carried by cations approximately in proportion to their concentrations in endolymph.  $\text{K}^+$ , the predominant ion, will normally carry most of the current. The channel is equally permeable to the other alkali cations, however, and moderately permeable to  $\text{Ca}^{2+}$  and to an organic cation, TMA. As the unhydrated diameter of TMA is  $0.54$  nm, the channel interior must be at least this large. It therefore resembles the nonspecific, acetylcholine-activated channel of the motor endplate, which has an internal diameter of at least  $0.65$  nm (ref. 19).

It remains to be seen whether the transduction channels of all vertebrate hair cells are comparably nonspecific. The fact that  $\text{Na}^+$  can support transduction in the goldfish sacculus<sup>7</sup> suggests that the channels in this organ are also nonspecific. The argument that ions other than  $\text{K}^+$  cannot carry transduction current in the mammalian cochlea rests on the observation that substitution of  $\text{Na}^+$  for  $\text{K}^+$  in cochlear endolymph gradually but irreversibly blocks the response<sup>6</sup>. This might, however, reflect secondary effects of  $\text{Na}^+$  accumulation on hair cells, such as the metabolic work required to pump this ion out of cells, rather than an inability of  $\text{Na}^+$  to carry receptor current into cochlear hair cells. Inasmuch as the hair cells of lower vertebrates share with those of the cochlea a transduction process operating in high- $\text{K}^+$  fluid, mediated by stereocilia<sup>10</sup>, and capable of great speed<sup>15</sup>, it seems likely that the transduction channel of cochlear hair cells is also nonspecific in its ionic selectivity.

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**Fig. 1** Platelet aggregation induced by ADP at 12  $\mu\text{M}$  (a) and 25  $\mu\text{M}$  (b), and its inhibition by AAG and AAG-D. In a, curve 1 represents the buffer control; curve 2 inhibition by 2  $\text{mg ml}^{-1}$  AAG; and curve 3 inhibition by 2  $\text{mg ml}^{-1}$  AAG-D. In b, curve 1 represents the buffer control; curve 2 inhibition by 2  $\text{mg ml}^{-1}$  AAG; curve 3 inhibition by 0.1  $\text{mg ml}^{-1}$  AAG-D; and curve 4 the inhibition observed in the presence of 0.5–2.0  $\text{mg ml}^{-1}$  AAG-D. AAG-D was substantially more inhibitory than the native molecule in all conditions tested.

## Inhibition of platelet aggregation by native and desialised alpha-1 acid glycoprotein

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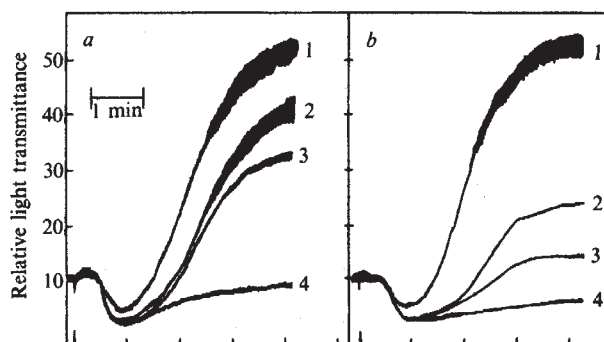
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The alpha-1 acid glycoprotein (orosomucoid; AAG) is a normal constituent of human plasma ( $650 \pm 215 \mu\text{g ml}^{-1}$ ) which increases in concentration as much as fivefold in association with acute inflammation and cancer, and thus is recognised as an acute phase protein<sup>1,2</sup>. AAG consists of a single polypeptide chain, has a molecular weight of 44,100, and contains ~45% carbohydrate including 12% sialic acid; it is the most negatively charged of the plasma proteins<sup>3</sup>. Certain of the biological properties of AAG are related to its sialic acid content<sup>1,3</sup>; thus, clearance and immunogenicity of AAG are markedly increased on desialisation<sup>4,5</sup>. The biological functions of AAG are largely unknown. AAG has the ability to inhibit certain lymphocyte reactivities including blastogenesis in response to concanavalin A, phytohaemagglutinin and allogeneic cells<sup>6</sup>, and these inhibitory effects are enhanced in association with desialisation<sup>7</sup>. In view of these observations, a report that unphysiologically large ( $5\text{--}15 \text{ mg ml}^{-1}$ ) amounts of AAG inhibit the platelet aggregation induced by ADP and adrenaline<sup>8</sup>, and evidence that a sialic acid-deficient species of AAG appears elevated in several chronic disease states<sup>9,10</sup>, we compared the effects of AAG and its desialised counterpart (AAG-D) on platelet aggregation. We report that desialisation of AAG is associated with increased expression of activity inhibitory to the platelet aggregation otherwise observed on stimulation with ADP, collagen or thrombin.

AAG was isolated from human pleural fluids as described previously<sup>6</sup>. The purity of the four AAG preparations tested was established by SDS-polyacrylamide gel electrophoresis, immunoelectrophoresis and column chromatography on Sephadex G-75 and DEAE-cellulose. Desialisation was performed using *Vibrio cholerae* neuraminidase according to Schmid<sup>11</sup>, with subsequent removal of the neuraminidase by reisololation of the AAG using DEAE-cellulose column chromatography; >75% loss of sialic acid<sup>12</sup> and 52% decrease of relative mobility on electrophoresis in polyacrylamide gels<sup>13</sup> were observed. Absence of residual neuraminidase activity in the AAG-D preparations was established by incubation with native AAG as the substrate using conditions optimal for enzyme activity; no neuraminidase activity was detected

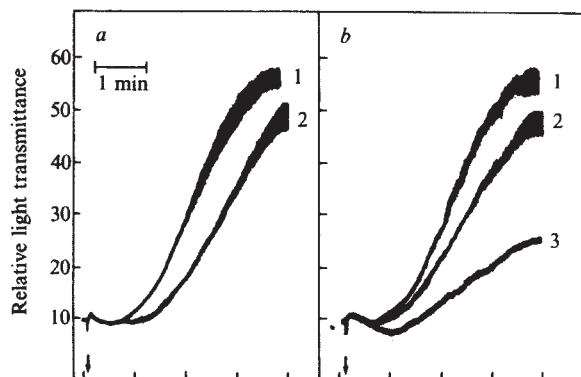
(<0.017 units per mg AAG-D). The standard platelet reaction mixtures consisted of 500  $\mu\text{l}$  platelet-rich plasma (PRP), 200  $\mu\text{l}$  of a dilution of AAG or AAG-D (in phosphate-buffered saline) and various amounts (in 10–15  $\mu\text{l}$ ) of the aggregating agent; the platelets were preincubated with AAG or AAG-D in the aggregometer for 30 s before stimulation with ADP, acid-soluble collagen<sup>14</sup>, or thrombin. In experiments involving platelet activation by thrombin, 500  $\mu\text{l}$  of a washed platelet suspension ( $3.0 \times 10^8 \text{ ml}^{-1}$ ) was used in place of PRP<sup>15</sup>.

The addition of either AAG or AAG-D ( $2 \text{ mg ml}^{-1}$ ) to PRP resulted in inhibition of the platelet aggregation otherwise observed on challenge with 12  $\mu\text{M}$  ADP (Fig. 1a). AAG-D was substantially more inhibitory than native AAG at all doses of the glycoprotein tested, with inhibition directly proportional to the concentration of AAG and AAG-D and inversely proportional to the intensity of the ADP challenge stimulus. Thus, on challenge with a larger amount (25  $\mu\text{M}$ ) of ADP, 2  $\text{mg ml}^{-1}$  AAG was no longer inhibitory, while AAG-D remained inhibitory even at concentrations as low as 50  $\mu\text{g ml}^{-1}$ ; concentrations of AAG-D to 2  $\text{mg ml}^{-1}$  did not induce substantially greater degrees of inhibition but did bring about deaggregation of the stimulated platelets (Fig. 1b). The inhibitory effects of AAG and AAG-D were directed preferentially against the secondary wave of platelet aggregation, although with low (4  $\mu\text{M}$ ) ADP stimulation a minimal inhibitory effect on the primary aggregation wave also was observed.



**Fig. 2** Platelet aggregation induced by 9  $\mu\text{g ml}^{-1}$  acid-soluble collagen and its inhibition by AAG (a) and AAG-D (b). In a, curve 1 represents the buffer control, while curves 2, 3 and 4 represent stimulation in the presence of 0.05, 0.5 and 2.0  $\text{mg ml}^{-1}$  AAG, respectively. In b, curve 1 represents the buffer control, and curves 2, 3 and 4 show stimulation in the presence of 0.05, 0.1 and 0.5  $\text{mg ml}^{-1}$  AAG-D, respectively. AAG-D again was substantially more inhibitory than the native molecule.





**Fig. 3** Platelet aggregation induced by  $0.014 \text{ U ml}^{-1}$  bovine topical thrombin and its inhibition by AAG (a) and AAG-D (b). In a, curve 1 represents the buffer control, and curve 2 represents stimulation in the presence of  $1 \text{ mg ml}^{-1}$  AAG. In b, curve 1 represents the buffer control, and curves 2 and 3 show stimulation in the presence of  $0.25$  and  $1 \text{ mg ml}^{-1}$ , respectively. AAG-D again was substantially more inhibitory than the native molecule.

Both AAG and AAG-D were also capable of inhibiting the platelet response to collagen, with AAG-D again much more inhibitory than the native molecule (Fig. 2). Thus, AAG-D at  $50\text{--}100 \mu\text{g ml}^{-1}$  markedly inhibited platelet aggregation, while native AAG was only minimally inhibitory at comparable concentrations; however, larger amounts of AAG ( $2 \text{ mg ml}^{-1}$ ) were fully inhibitory. Inhibition was characterised by decreased rates and extents of aggregation with a minimal effect on the onset of platelet shape change. The addition of AAG and particularly of AAG-D also resulted in decreased secretion of platelet serotonin on stimulation with ADP or collagen.

AAG and AAG-D also inhibited platelet aggregation induced by thrombin and AAG-D was again the more inhibitory with  $250 \mu\text{g ml}^{-1}$ , compared to  $1 \text{ mg ml}^{-1}$  of native AAG, needed for significant inhibition (Fig. 3). Decreased inhibition was observed when the concentration of the thrombin challenge dose was increased. In controls for the above experiments platelet viability was unaltered as monitored by  $^{51}\text{Cr}$  release, and thus could not account for the inhibitory effects observed. Further, the addition of neuraminidase in amounts ( $0.03\text{--}0.09$  units  $\text{ml}^{-1}$ ) up to fivefold greater than the minimum detectable in the assays used in this work had either no effect on, or enhanced, both ADP- and thrombin-induced platelet aggregation.

The mechanism(s) by which AAG inhibits the platelet response to ADP, collagen and thrombin is not yet known, nor is the manner in which desialisation seems to evoke further or enhance this inhibitory activity. Thus, removal of sialic acid residues may directly alter the exposure or effectiveness of appropriate reactive sites or indirectly favour a further critical modification of AAG such as its aggregation<sup>16</sup>. Moreover, we do not yet know the relative activities of the polymorphic or altered forms of AAG that have been observed in various sera and fluids<sup>3,9,10</sup>. Nonetheless, it is clear that both native and desialised AAG share with another acute phase protein, the C-reactive protein or CRP, the capacity to inhibit platelet responsiveness<sup>14,15</sup>, and like CRP the effects of AAG are directed predominantly to events critical for the secondary wave of platelet aggregation. The present results thus point to AAG as a potentially significant modulator of platelet responsiveness, suggest desialisation or an associated alteration of the molecule as a mechanism by which this biological potential is evoked and/or enhanced, and support the hypothesis that the acute phase response represents, at least in part, a coordinated system to limit, modulate and/or otherwise direct host immune responses during periods of intense inflammation and tissue destruction.

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## Cell-mediated immunity to *Theileria*-transformed cell lines

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In East and Central Africa the protozoan parasite *Theileria parva* causes a disease of cattle called East Coast fever (ECF). In Kenya alone between 60,000 and 85,000 cattle die from ECF every year<sup>1</sup>. Infected animals can recover from ECF either naturally<sup>2</sup> or after treatment with tetracyclines<sup>3</sup> or menotone<sup>4</sup> and are subsequently able to resist challenge with the homologous strain of parasite. That this acquired resistance is due to cell-mediated rather than humoral immunity has been suspected<sup>5,6</sup> but never decisively shown. A major difficulty in studying immunity to ECF has been the lack of inbred animals for studying *Theileria*-specific immunity in the absence of allogeneic histocompatibility barriers. We have avoided this problem by measuring cell-mediated immune responses in a syngeneic system *in vitro*. Unidirectional mixed lymphocyte cultures (MLC) were set up using bovine peripheral blood lymphocytes (PBL) as responder cells and autologous cell lines transformed *in vitro* by *T. parva* as stimulator cells. In these cultures, DNA synthesis was induced in PBL from both normal and *Theileria*-immune animals. However, cytotoxic lymphocytes were induced only in cultures containing responder lymphocytes from *Theileria*-immune cattle. The results show that *Theileria*-transformed cells express antigens which are recognised by effector cells and provide evidence that cell-mediated cytotoxic mechanisms function in immunity to ECF.

By culturing cell suspensions from lymph nodes, spleen or peripheral blood of *Theileria*-infected cattle, transformed lymphoblastoid cell lines can be obtained<sup>7</sup>. Such cell lines can also be obtained by infecting normal bovine lymphocytes *in vitro* with *Theileria* sporozoites<sup>8</sup>. It is likely, therefore, that these cell lines represent the schizogonous stage of the parasite in the host animal which is characterised by the presence of macroschizonts in the transformed lymphocyte. Of more than 10 different *Theileria*-transformed cell lines examined by immunofluorescence, all were surface immunoglobulin negative and stained positively with fluoresceinated peanut agglutinin (G. E. Roelants *et al.* unpublished). Thus we believe that the *Theileria*-infected cells are bovine T lymphocytes<sup>9</sup>.

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**Table 1** Stimulation of bovine lymphocytes by syngeneic *Theileria*-transformed cell lines

| Cell combination                            | Mean<br>c.p.m. | Maximum<br>stimulation ratio | Cell combination                            | Mean<br>c.p.m. | Maximum<br>stimulation ratio |
|---------------------------------------------|----------------|------------------------------|---------------------------------------------|----------------|------------------------------|
| A PBL <sub>784</sub> × medium               | 117            | —                            | B PBL <sub>951</sub> × medium               | 241            | —                            |
| PBL <sub>784</sub> × PBL <sub>784(R)</sub>  | 105            | —                            | PBL <sub>951</sub> × PBL <sub>951(R)</sub>  | 252            | —                            |
| LINE <sub>784(R)</sub> × medium             | 74             | —                            | LINE <sub>951(R)</sub> × medium             | 286            | —                            |
| PBL <sub>784</sub> × LINE <sub>784(R)</sub> | 25,039         | 238.5                        | PBL <sub>951</sub> × LINE <sub>951(R)</sub> | 16,703         | 66.3                         |
|                                             |                |                              | PBL <sub>989</sub> × PBL <sub>951(R)</sub>  | 5,061          | 21.0                         |

Lymphocytes were separated from heparinised peripheral blood of female Friesian animals 951 and 784 by centrifugation through Ficoll-Paque (Pharmacia) and adjusted to  $2.5 \times 10^6$  cells per ml in RPMI-1640 medium containing heat-inactivated fetal calf serum (5%), glutamine (2mM), 2-mercaptoethanol ( $5 \times 10^{-5}$  M), penicillin ( $100 \mu\text{g ml}^{-1}$ ), streptomycin ( $100 \text{ IU ml}^{-1}$ ) and HEPES buffer (10mM)<sup>9</sup>. Volumes of 0.1 ml were dispensed in triplicate into wells of Falcon 3040 flat-bottomed microplates. One hundred  $\mu\text{l}$  of medium alone or of a suspension of irradiated syngeneic *Theileria*-transformed cells ( $2.5 \times 10^5$  cells  $\text{ml}^{-1}$ ) were added to the appropriate microwells. The subscript (R) indicates that the cells were irradiated with 5,000 R from a caesium source. The microplates were incubated at 37 °C in 5% CO<sub>2</sub> in air and at 48, 96, 144 and 192 h, 0.5  $\mu\text{Ci}$  <sup>125</sup>IUDR was added to each well. Sixteen hours later, cultures were collected onto glass fibre filters using a Skatron multiple sample harvester. Filters were dried under an IR lamp and counted for 1 min in a  $\gamma$  counter. Mean counts per minute were determined and stimulation ratios calculated as mean c.p.m. in PBL-syngeneic line combinations/mean c.p.m. in PBL-syngeneic PBL combinations. Stimulation ratios were determined for each time point but only the maximum stimulation ratios were shown here (peak was at day 6 for both experiments shown). In preliminary experiments, eight batches of fetal calf sera were tested at 5, 10 and 20% final concentration. One batch (used at 5% final concentration) gave the highest stimulation ratios and was used in all further experiments. Various cell concentrations, stimulator: responder cell ratios and irradiation doses were also tested and optimal conditions determined before starting the experiments presented in this paper. The data shown are representative of data obtained in four experiments performed with PBL and cell line from animal 784 and in six experiments with PBL and cell line from animal 951. The cell lines used as stimulators were derived by infecting lymphoid cells *in vitro* with sporozoites of *T. parva* from ground up ticks using the method of Brown *et al.*<sup>8</sup>. Lymph nodes were removed from Friesian heifers 951 and 784 and their cells infected from a common pool of *T. parva* (Muguga). The lymphoblastoid lines were maintained by twice weekly passage in Eagle's minimal essential medium with Earle's salts and 20% fetal calf serum. For use in MLC and for generation of cytotoxic lymphocytes, the cell lines were transferred and grown in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum and were subcultured every two days to ensure healthy cells. All cattle used were judged to be healthy by the veterinary staff at the Kenya Agricultural Research Institute. Animals which were known to be *Theileria*-immune had high levels of anti-macroschizont antibodies (detected by indirect immunofluorescence) in their sera. Those which were considered non-immune were clinically normal and their sera contained no detectable anti-macroschizont antibodies.

It is thought that host cells must be infected with *Theileria* parasites before immunity can be achieved<sup>10</sup>. This led us to believe that the infected lymphocytes carry infection- or transformation-associated antigens on their surface and that these antigens are recognised by the host immune system. To test for such antigens, DNA synthesis was measured in MLCs containing syngeneic responder-stimulator combinations as shown in Table 1. *Theileria*-transformed cells stimulated <sup>125</sup>I-iododeoxyuridine (<sup>125</sup>IUDR) uptake in autologous PBL from normal or *Theileria*-immune animals and with the same kinetics in each case. Normal PBL were stimulated to similar degrees with autologous and allogeneic *Theileria*-transformed stimulator cells (data not shown). The stimulation ratio in cultures containing the allogeneic PBL combination (PBL<sub>784</sub> × PBL<sub>951(R)</sub>) was much lower than that obtained when the allogeneic *Theileria*-transformed cells were used as stimulators suggesting that the high stimulation was caused by special properties of the transformed cells and not simply by differences in normal histocompatibility antigens. Autoradiography showed that DNA synthesis was increased in the nuclei of responder lymphocytes (G. E. Roelants and T.P., unpublished). This sti-

**Table 3** The effect of heating and drug treatment of *Theileria*-transformed stimulating cells in MLR

| Cell combination                                         | Mean<br>c.p.m. | Stimulation<br>ratio |
|----------------------------------------------------------|----------------|----------------------|
| PBL <sub>951</sub> × PBL <sub>951(R)</sub>               | 340            | 1.0                  |
| PBL <sub>951</sub> × LINE <sub>951(R)</sub>              | 2,860          | 8.4                  |
| PBL <sub>951</sub> × LINE <sub>951(R)</sub> 993C treated | 407            | 1.2                  |
| PBL <sub>951</sub> × LINE <sub>951(R)</sub> heat treated | 238            | 0.7                  |

Cell combinations were set up in microplates as described in Table 1 legend. Some stimulator cells were heat-killed (as assessed by Trypan blue exclusion) by incubating at 56 °C in a water bath for 45 min. Drug treatment of stimulator cells was performed by incubating  $5 \times 10^7$  *Theileria*-transformed cells at 37 °C for 48 h with  $1 \mu\text{g ml}^{-1}$  final concentration of the compound 993C after which they were washed three times. The cells were 94% viable after treatment. The stimulation of DNA synthesis was assessed at days 3, 4, 5 and 6. Only the results from day 6 are shown here although the same pattern of stimulation was seen at all time points. The data shown are representative of data obtained in three experiments.

**Table 2** The effect of MLR culture supernatants on DNA synthesis in bovine lymphocytes

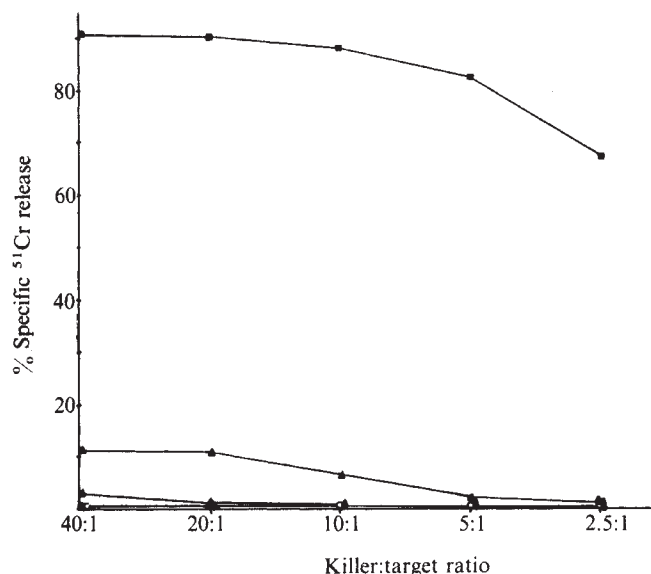
| Cell combination/supernatant                                 | Mean<br>c.p.m. | Stimulation<br>ratio |
|--------------------------------------------------------------|----------------|----------------------|
| A PBL <sub>951</sub> + fresh medium                          | 324            | —                    |
| + supernatant 1                                              | 331            | —                    |
| + supernatant 2                                              | 361            | —                    |
| + supernatant 3                                              | 301            | —                    |
| + supernatant 4                                              | 302            | —                    |
| + supernatant 5                                              | 302            | —                    |
| B PBL <sub>951</sub> × LINE <sub>951(R)</sub> + fresh medium | 2,568          | 12.7                 |
| + supernatant 1                                              | 2,201          | 10.9                 |
| + supernatant 2                                              | 2,201          | 11.4                 |
| + supernatant 3                                              | 1,999          | 9.9                  |
| + supernatant 4                                              | 2,109          | 10.5                 |
| + supernatant 5                                              | 1,990          | 9.9                  |

Various cell combinations were set up in microplates following the procedure outlined in Table 1 legend. After incubation for 5 days, supernatants were removed from the culture wells and 100- $\mu\text{l}$  aliquots (either neat or after concentrating fivefold with an Amicon UM-2 filter) were added to microwells containing normal PBL (A) or mixed lymphocyte cultures (B). Six days later, <sup>125</sup>IUDR was added to the microwells and the well contents collected as described in Table 1 legend. Supernatants were prepared from the following cell combinations: (1) PBL<sub>951</sub> × PBL<sub>951(R)</sub> (2) PBL<sub>951</sub> × LINE<sub>951(R)</sub> (3) PBL<sub>951(R)</sub> × LINE<sub>951(R)</sub> (4) LINE<sub>951(R)</sub> × medium (5) LINE<sub>951</sub> × medium. In experiment B, the control culture (PBL<sub>951</sub> × PBL<sub>951(R)</sub>) showed 201 mean c.p.m.

mulation was probably caused by antigens on the surface of viable stimulating cells since culture supernatants from MLCs had no effect on DNA synthesis of responder cells (Table 2). Addition of freshly prepared free macroschizonts to PBL cultures also failed to stimulate DNA synthesis. We did not detect schizonts in dividing cells in Giemsa stained preparations from 6–10 day MLCs showing that transformation of normal PBL had not occurred. Transformed cells, killed by heating or treated with the compound 993C (a 2-cycloalkyl-3-hydroxy-1, 4-naphthoquinone, Wellcome) to eliminate the schizonts, failed to stimulate DNA synthesis in MLC (Table 3). Compound 993C is selectively toxic for the macroschizont in the transformed cell and thereby stops parasite-induced proliferation but leaves the cells in a viable but non-transformed state<sup>11</sup>. These results suggest that antigens present on the surface of *Theileria*-transformed cells are involved in stimulation of normal syngeneic PBL and that the antigens are *Theileria*-associated and not due solely to culture *in vitro*. In addition, the heat-treatment experiment shows that viable stimulator cells are required, a feature of a true mixed lymphocyte reaction (MLR)<sup>12</sup>.

A positive MLR represents the cognitive and proliferative phase of an immune response<sup>13</sup>. However, proliferation of cells is not in itself evidence for functional immunity. For this reason, we tested for cell-mediated lysis of *Theileria*-transformed cell lines by using cells from MLCs as effectors in <sup>51</sup>Cr-release assays (Fig. 1). Cytotoxic activity was only detected after secondary stimulation *in vitro* of PBL from immune animal 713. Cytotoxic





**Fig. 1** Cell-mediated lysis of *Theileria*-transformed cell lines. Bovine peripheral blood lymphocytes were purified<sup>9</sup> from non-immunised animal 951 or from *Theileria*-immune animal 713 and cytotoxic lymphocytes were generated in MLC and assayed as previously described for the mouse<sup>16</sup>, except that the stimulator cells were irradiated (5,000R from a caesium source), the MLCs were incubated for 6 days, and the cytotoxic cells were adjusted to  $1 \times 10^7$  per ml. Animal 713 was immunised by infecting with *T. parva* sporozoites and treating with the compound 993C 11 days later. It was then injected at 2-month intervals with  $1 \times 10^9$  cells of the autologous *Theileria*-transformed line 713 on three separate occasions. The *Theileria*-transformed cells were labelled with  $^{51}\text{Cr}$  (ref. 17) and used as targets within 30 min of labelling. Background (medium) release was 9% for the LINE<sub>713</sub> and 10% for the LINE<sub>951</sub>. The data shown are representative of data from nine independent experiments with effectors from PBL<sub>713</sub> and LINE<sub>713</sub> MLC combinations and four experiments using cells from PBL<sub>951</sub> and LINE<sub>951</sub> combinations.

**Effector:**

- PBL<sub>713</sub> (directly out of animal)
  - △ PBL<sub>713</sub> × MEDIUM
  - PBL<sub>713</sub> × LINE<sub>713(R)</sub>
  - PBL<sub>951</sub> × LINE<sub>951(R)</sub>
  - ▲ PBL<sub>713</sub> × LINE<sub>713(R)</sub>
- } On LINE<sub>713</sub> targets.
- } On LINE<sub>951</sub> targets.

activity was also seen when effectors from PBL<sub>A143</sub> × LINE<sub>A143.D7(R)</sub> and from PBL<sub>953</sub> × LINE<sub>953(R)</sub> MLC combinations were used on homologous targets. The PBL<sub>A143</sub> were from a bovid which was infected with sporozoites and cured by treatment with tetracyclines and PBL<sub>953</sub> were from an animal which became naturally infected and was cured with menotone. A low level of killing of allogeneic *Theileria*-transformed cell lines was observed with each of these effector populations. No killing was observed when effectors were from MLCs containing PBL from normal animals 951 (Fig. 1) or A149 (data not shown). It is likely that these PBL require an initial 'priming' in order to generate helper cells for cytotoxic cells or to generate cytotoxic cell precursors. In the generation of cytotoxic T lymphocytes in the murine system<sup>14</sup>, proliferation of 'helper' or precursor lymphocytes occurs on initial exposure to antigen. It is possibly this type of proliferation that we measured in the primary MLRs. It follows that after secondary stimulation *in vitro*, as with PBL<sub>713</sub>, PBL<sub>A143</sub> and PBL<sub>953</sub> which were taken from immune animals, enough 'helper' cells or cytotoxic cell precursors were present to allow differentiation of cytotoxic effectors.

Our results show that cytotoxic cells can be induced in PBL from *Theileria*-immune animals by co-culture with syngeneic *Theileria*-transformed cell lines. These cytotoxic cells kill *Theileria*-transformed cells and their generation may thus be used as a measure of immunity to the parasites. It is most likely that after challenge with *Theileria*, cytotoxic cells develop in both immune and non-immunised cattle. Why then do most non-immunised cattle die after between 18 and 24 days of

infection? Our results support the widely held hypothesis<sup>15</sup> that it is simply due to outpacing of the immune response by growth of the parasite since once an animal has recovered from infection it is solidly immune. By biasing the immune system towards *Theileria*-specific cell-mediated immunity it should be possible to immunise cattle. Our results suggest that priming of the bovine immune system with allogeneic *Theileria*-transformed cell lines may be possible, that is, the priming may not be H-restricted. If such cell lines are attenuated so that they do not cause lethal infection they may allow sensitisation of the immune system so that a second exposure to antigen causes an effective cytotoxic response to *Theileria*-transformed lymphocytes.

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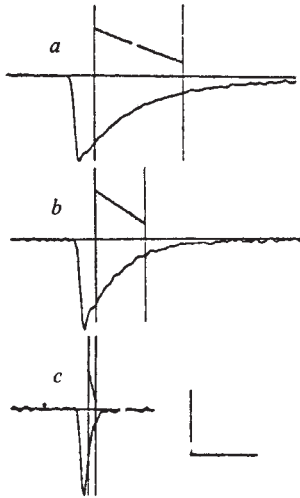
## Clindamycin and lincomycin alter miniature endplate current decay

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**Antibiotic-induced muscle paralysis has frequently been found in both experimental animals and man with three distinct classes of antibiotic: (1) streptomycin and related aminoglycoside compounds, (2) polymyxins and (3) tetracyclines<sup>1</sup>. Recently lincomycin and its chemical congener, clindamycin, have been reported to produce muscle paralysis which has different characteristics from those seen with other classes of antibiotic<sup>2–4</sup>. Although closely related in chemical structure, lincomycin and clindamycin also seem to produce muscle paralysis by different mechanisms. Clindamycin is considered to exert a direct depressant action on muscle contractility whereas the action of lincomycin is considered to be primarily a depression of neuromuscular transmission. We report here that each of these antibiotics had a significant but different influence on endplate channel behaviour. Clindamycin increased the rate of miniature endplate current (m.e.p.c.) decay and reduced its voltage sensitivity without altering its exponential nature. Lincomycin split m.e.p.c. decay into an initial rapid phase followed by a prolonged phase.**

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**Fig. 1** The effect of clindamycin on m.e.p.c. decay is concentration dependent. Averaged m.e.p.cs (13–17 individual currents) obtained in a fibre voltage clamped at  $-120$  mV in control Ringer (a)  $2.12 \times 10^{-5}$  M clindamycin (b) and  $4.26 \times 10^{-4}$  M clindamycin (c). Above each current record a portion of the decay is plotted semilogarithmically and fitted with a least-squares regression line to evaluate the decay time constant. The linearity of the plot indicates that m.e.p.c. decay as a single exponential with time. The decay time constants (ms) were: a, 3.41; b, 1.89; c, 0.298. Calibration bars: a, 5.4 nA, 3.5 ms; b, c, 2.7 nA, 3.5 ms.

Spontaneous m.e.p.cs were recorded in voltage-clamped twitch fibres of the costocutaneous muscle of garter snakes (*Thamnophis* sp.). Muscles were removed with a portion of the rib and scale on which they inserted and mounted in a small (4 ml) Plexiglass chamber filled with physiological solution of composition (mM): NaCl 159; KCl 2.15;  $\text{CaCl}_2$  2.0;  $\text{MgCl}_2$  4.2; HEPES 1.0, at pH 7.3–7.4. Experiments were carried out at room temperature ( $20$ – $24^\circ\text{C}$ ). All solutions contained 100 nM tetrodotoxin. The snake neuromuscular junction is well suited for these studies, for the endplate region is compact relative to the fibre space constant ( $1$ – $4$  mm)<sup>5</sup>, enabling the voltage clamp to provide effective voltage control over the entire active synaptic area. With the muscle spread laterally, individual endplates can be easily visualised and voltage clamped with two micro-electrodes as described previously<sup>6</sup>. M.e.p.cs were digitally recorded and analysed using a DEC PDP8E computer; those with the most rapid rate of rise ( $<1$  ms) were selected for analysis. An average of 10–20 m.e.p.cs were recorded and averaged at each holding potential. Stock solutions of the antibiotics (clindamycin HCl, lincomycin HCl) were freshly prepared in the physiological solution. Drugs were added by replacing the bath volume three times with drug-containing solution. An equilibration period of at least 10 min elapsed before recording at each concentration. Averaged control m.e.p.cs could be fitted to the expression,  $I(t) = I(0) \exp(-t/\tau)$ , where  $I(0)$  is the peak current,  $t$  is time after peak and  $\tau$  is the decay time constant given by the semilogarithmic slope. At all voltages control m.e.p.cs decayed with a single exponential time course. The decay time constant was determined by confining the analysed portion to between 20 and 80% of the peak amplitude.

Clindamycin reduced the amplitude and shortened the time course of m.e.p.c. decay. The effects of clindamycin were concentration dependent in the range studied ( $2.12 \times 10^{-5}$  M– $4.26 \times 10^{-4}$  M). Figure 1 shows the concentration dependence in a single fibre voltage clamped at  $-120$  mV. Note that both in control records and records of the two concentrations of clindamycin the m.e.p.cs decayed as a single exponential with time.

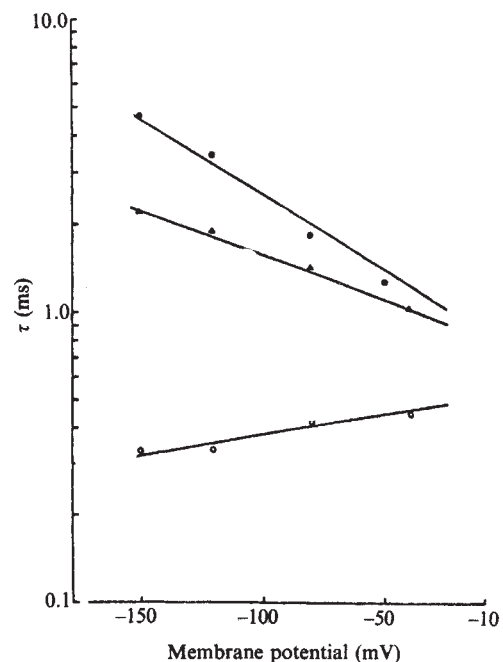
This was consistently observed at all voltages and concentrations of clindamycin. The dependence of the m.e.p.c. decay on clindamycin concentration is indicated by the amount of change in the semilogarithmic plot above each current record. In Fig. 1  $\tau$  was decreased 45% and 91% in clindamycin concentrations of  $2.12 \times 10^{-5}$  M and  $4.26 \times 10^{-4}$  M, respectively.

In normal physiological solution  $\tau_{\text{m.e.p.c.}}$  is sensitive to changes in membrane potential<sup>6–8</sup>. The voltage dependence of  $\tau_{\text{m.e.p.c.}}$  can be accurately described by the exponential function

$$\tau(V) = \tau(0)e^{-AV} \quad (1)$$

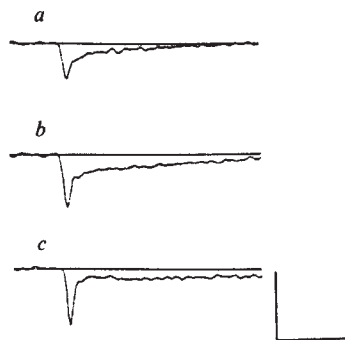
where  $\tau(0)$  is the decay time constant at zero membrane potential,  $V$  is the membrane potential and  $A$  is the voltage coefficient of  $\tau$ . In five control cells the average coefficient of voltage sensitivity,  $A$  ( $\text{mV}^{-1}$ ), was  $0.015 \pm 0.001$  (mean  $\pm$  s.e.). This value is similar to that previously reported for snake twitch fibres<sup>6</sup>. The dependence of  $\tau$  on voltage obtained in a single fibre for both control and two concentrations of clindamycin is illustrated in Fig. 2. Values of  $\tau(0)$  and  $A$  calculated according to equation (1) were: control physiological solution:  $\tau(0) = 0.783$  ms,  $A = -0.0118 \text{ mV}^{-1}$ ;  $2.12 \times 10^{-5}$  M clindamycin:  $\tau(0) = 0.775$  ms,  $A = -0.0069 \text{ mV}^{-1}$ ;  $4.26 \times 10^{-5}$  M clindamycin:  $\tau(0) = 0.521$ ,  $A = +0.0033 \text{ mV}^{-1}$ . In the presence of clindamycin there was a concentration-dependent reduction in the voltage sensitivity. With concentrations of clindamycin of  $2.12 \times 10^{-4}$  M or higher the voltage sensitivity was either abolished or reversed (as in Fig. 2). The shortening of m.e.p.c. decay and reduction in the voltage sensitivity of  $\tau_{\text{m.e.p.c.}}$  produced by clindamycin were reversible on return to control physiological solution.

In marked contrast to the effects of clindamycin on m.e.p.c. decay lincomycin converted the decay of m.e.p.cs to a multi-exponential function consisting of at least two distinct components, one more rapid and another slower than the decay of control m.e.p.cs. The effect of lincomycin on m.e.p.c. decay rates and amplitude was both voltage and concentration



**Fig. 2** The voltage dependence of  $\tau$  is reduced by clindamycin. m.e.p.c. decay for both control and clindamycin depend exponentially on voltage over the range  $-40$  mV to  $-150$  mV. Control (●) and measurements in the presence of clindamycin,  $2.12 \times 10^{-5}$  M (▲) and  $4.26 \times 10^{-4}$  M (○), were fitted according to equation (1).





**Fig. 3** The action of lincomycin on m.e.p.c. decay is voltage dependent. Averaged m.e.p.c.s (13–20 individual currents) were recorded in a single fibre at  $-50$  mV (a),  $-100$  mV (b) and  $-150$  mV (c). Lincomycin concentration was  $4.43 \times 10^{-4}$  M. Calibration bars: 2.7 nA, 3.5 ms.

dependent. Increasing the concentration of lincomycin above the threshold concentration ( $10^{-4}$  M) at a fixed voltage produced an increase in the relative size of the rapid initial decay and prolonged the time course of the terminal phase. Similarly, these phases varied with membrane potential at a constant lincomycin concentration. The voltage dependence of lincomycin action recorded in a single voltage-clamped fibre is shown in Fig. 3. The initial fast component of decay declined more quickly and comprised a larger portion of the total m.e.p.c. decay as the membrane was hyperpolarised. The slow component became greatly reduced in amplitude and decayed very slowly with hyperpolarisation. The time constants of decay for both phases were determined using a computerised least-squares exponential fitting program<sup>9</sup>. For the currents depicted in Fig. 3 the fast and slow decay time constants (ms) were, respectively, 0.30 and 3.56 at  $-50$  mV, 0.17 and 6.78 at  $-100$  mV, 0.17 and 20.8 at  $-150$  mV.

The time constant of m.e.p.c. decay is well established as a measure of the mean open duration of endplate channels in frog twitch fibres<sup>7</sup>. A similar relationship has been reported for snake twitch fibres<sup>6</sup>. Alteration in the decay of both e.p.c.s and m.e.p.c.s have been reported for various drugs, including local anaesthetics<sup>10–12</sup> and belladonna alkaloids<sup>13,14</sup>. The results reported here for clindamycin are similar to those previously reported for atropine and the lignocaine derivative QX314 (refs 10, 11). In addition, the splitting of m.e.p.c.s by lincomycin is qualitatively similar to results reported for scopolamine<sup>15</sup> and the lignocaine derivative QX222 (refs 10, 11). These similarities are consistent with an interaction of the antibiotics with the open state of the acetylcholine receptor-channel complex, creating a channel with little or no conductance<sup>12,15</sup>.

Clindamycin differs from lincomycin in chemical structure by the presence of a chloride substituent in position 7. The present results indicate that this chemical modification, which greatly increases the lipid solubility, influences the characteristics of m.e.p.c. decay. Of particular importance is the finding that these agents modify channel behaviour in concentrations which might normally be present in plasma. We suggest that the interaction of antibiotics with the endplate receptor-channel complex might help to explain the differences in neuromuscular block obtained with these antibiotics and also improve our understanding of the mechanisms of antibiotic-induced paralysis.

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## Role of intracellular $\text{Ca}^{2+}$ sequestration in $\beta$ -adrenergic relaxation of a smooth muscle

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Various mechanisms have been proposed for  $\beta$ -adrenergically mediated relaxation of smooth muscle. All theories suggest the involvement of cyclic AMP as a second messenger:  $\beta$ -agonists stimulate adenylate cyclase which converts ATP to cyclic AMP<sup>1</sup> and protein kinase, activated by cyclic AMP, is then thought to catalyse a protein phosphorylation that leads to a reduction in free  $\text{Ca}^{2+}$ , thus effecting relaxation<sup>1</sup>. How this last step is accomplished is much debated, but the following possibilities are currently considered as the mechanisms responsible for cyclic AMP-induced reduction of cytoplasmic  $\text{Ca}^{2+}$ : activation of a  $\text{Ca}^{2+}$ -ATPase in the plasma and/or sarcoplasmic reticulum membranes which lowers cytoplasmic  $[\text{Ca}^{2+}]$  in a direct manner or stimulation of  $(\text{Na}^+-\text{K}^+)\text{ATPase}$  in the cell membrane which may indirectly effect  $\text{Ca}^{2+}$  extrusion<sup>2–8</sup>. Among the hypotheses suggested, those of  $\text{Ca}^{2+}$  sequestration by the sarcoplasmic reticulum and of  $\text{Ca}^{2+}$  extrusion across the cell membrane are consistent with each other if it is assumed that both processes are effected by a cyclic AMP-sensitive  $\text{Ca}^{2+}$ -ATPase. However, quite a different mechanism is implied by involving the  $\text{Na}^+-\text{K}^+$  pump and  $\text{Na}^+-\text{Ca}^{2+}$  exchange carrier. In this report, we present evidence that suggests intracellular  $\text{Ca}^{2+}$  sequestration is the mechanism involved.

Stopping the  $\text{Na}^+-\text{K}^+$  pump with ouabain initiates a transient contraction in the taenia coli. The inhibited pump is unlikely to be stimulated by  $\beta$ -adrenergic activation, so this system provides the opportunity to test whether  $(\text{Na}^+-\text{K}^+)\text{ATPase}$  is required in cyclic AMP-mediated relaxation. The possible involvement of a  $\text{Na}^+-\text{Ca}^{2+}$  exchange carrier can be tested by studying relaxation of a high  $\text{K}^+$ -induced contracture in the absence of  $\text{Na}^+$ . In Fig. 1a,  $\beta$ -stimulation, phosphodiesterase inhibition and the application of 8-bromo-cyclic AMP can be seen to relax the ouabain-induced contraction. Each of these has been postulated to relax smooth muscle by increasing protein kinase activity through a rise in cytoplasmic cyclic AMP. In each case, the muscle relaxed below the resting tension. The delayed response of 8-bromo-cyclic AMP may be due to its slower diffusion into the cell. It is unlikely that these results are due to incomplete blockade of the  $\text{Na}^+-\text{K}^+$  pump because they were duplicated with a contraction initiated by  $10^{-4}$  M ouabain in the absence of external  $\text{K}^+$ .

Each of these agents also relaxed 145 mM  $\text{K}^+/0$   $\text{Na}^+$  contractures (Fig. 1b), although the isoprenaline relaxation was transient. The decline in tissue  $\text{Na}^+$  was measured in the 145 mM  $\text{K}^+/0$   $\text{Na}^+$  solution. After exposure for 0, 2, 5 and 10 min, respective tissue  $\text{Na}^+$  concentrations were  $60 \pm 3$ ,  $14.4 \pm 1.2$ ,  $4.9 \pm 0.9$  and 0 mM  $\text{Na}^+$  per kg taenia coli. At the point

when relaxing agents were added (3 min), not all the  $\text{Na}^+$  had left the cells. However, depletion of extracellular  $\text{Na}^+$  reversed its gradient and would thus be unable to effect  $\text{Ca}^{2+}$  extrusion as postulated by Scheid *et al.*<sup>7</sup>. Identical relaxations were also obtained after exposure to the 145 mM  $\text{K}^+$ /0  $\text{Na}^+$  solution for 30 min, when all  $\text{Na}^+$  had left the tissue.

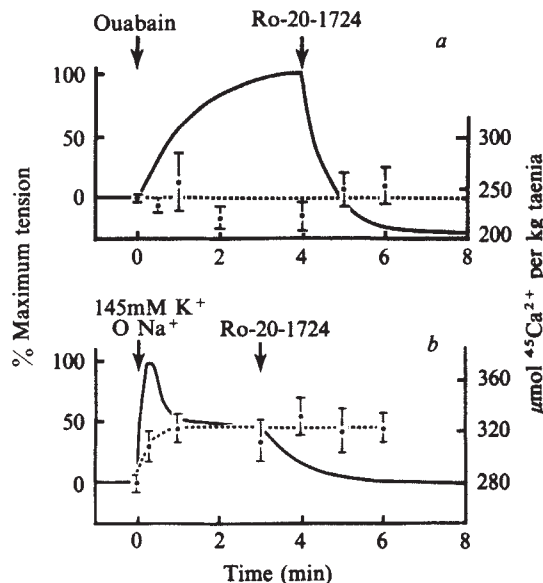
Net  $\text{Ca}^{2+}$  fluxes (Fig. 2a, b) were measured during phosphodiesterase inhibition, as R0-20-1724 (a phosphodiesterase inhibitor; Roche) induced the most rapid tension reductions (see Fig. 1b). Neither ouabain nor the subsequent addition of R0-20-1724 induced significant changes in total exchangeable tissue  $\text{Ca}^{2+}$  (Fig. 2a). These results indicate that  $\text{Ca}^{2+}$  is released intracellularly during ouabain contraction and sequestered by an intracellular store during phosphodiesterase-induced relaxation.

The high- $\text{K}^+$  contraction (Fig. 2b) is characterised by an initial phasic portion that has been postulated to be at least partly mediated by intracellular  $\text{Ca}^{2+}$  release<sup>9,10</sup>. This is followed by a tonic contraction maintained by a net influx of  $\text{Ca}^{2+}$ . Again, however, no change in  $\text{Ca}^{2+}$  is observed with relaxation.

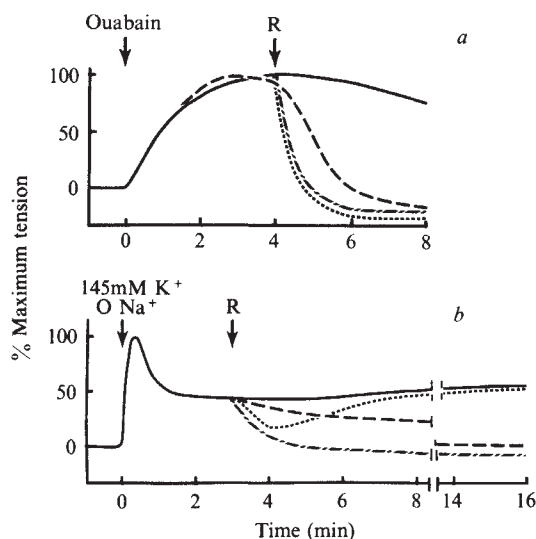
Relaxation by all three procedures, which raise cellular cyclic AMP, has been shown to occur when the  $\text{Na}^+-\text{K}^+$  pump is inoperative or when there is no  $\text{Na}^+$  in the medium or the cells. These results indicate that an increased inward-directed  $\text{Na}^+$  gradient, whether or not the result of  $\text{Na}^+-\text{K}^+$  pump stimulation, is not essential for  $\beta$ -adrenergic relaxation.

A recent study<sup>7</sup> has reported that  $\text{Na}^+-\text{K}^+$  pump inhibition decreased the relaxing effect of isoprenaline or carbachol contractions. This could also be explained by superimposition of the ouabain contraction on the contractile effect of the carbachol.

The data presented here support the hypothesis that cyclic AMP-mediated relaxation is caused by  $\text{Ca}^{2+}$  sequestration<sup>4,11,12</sup>.



**Fig. 2** *a*, Net  $\text{Ca}^{2+}$  content of taenia coli during ouabain-induced contraction and subsequent R0-20-1724-induced relaxation. Tissues were incubated in  $^{45}\text{Ca}$ -labelled PSS for 90 min to label tissue  $\text{Ca}^{2+}$ ; they were then sequentially exposed to the contracting and relaxing solutions as in Fig. 1 which were labelled at the same specific radioactivity. Labelled  $\text{Ca}^{2+}$  was measured in a scintillation counter after removal of extracellular  $\text{Ca}^{2+}$  in ice-cold PSS (0  $\text{Ca} + 2$  mM EGTA) for 45 min. Points represent mean  $\pm$  s.e. for four determinations. *b*, Net  $\text{Ca}^{2+}$  during high  $\text{K}^+$ -induced contraction and subsequent relaxation by R0-20-1724. Tension tracing from *a*. Tissues were placed in 145 mM  $\text{K}^+$ -0  $\text{Na}^+$  HEPES buffer and R0-20-1724 (1 mM) was added 3 min later.  $^{45}\text{Ca}$  measurements were made as in *a*.



**Fig. 1** *a*, Relaxation of ouabain-induced contractions. Tension of pieces of taenia coli ~1 cm long was measured isometrically in physiological saline solution (PSS) (in mM;  $\text{NaCl}$ , 140;  $\text{KCl}$ , 5;  $\text{CaCl}_2$ , 1.5;  $\text{MgCl}_2$ , 1; dextrose, 10; HEPES, 5; pH 7.2, oxygenated) to which atropine ( $10^{-6}$  M) and phentolamine ( $10^{-5}$  M) had been added. These blocked any possible muscarinic or  $\alpha$ -adrenergic effects, respectively. Muscles were contracted with  $2 \times 10^{-5}$  M ouabain. Four minutes later the relaxants were added:  $5 \times 10^{-4}$  M 8-bromo-cAMP (---),  $10^{-3}$  M R0-20-1724 (- - -) or  $10^{-5}$  M isoprenaline (· · · · ·). *b*, Relaxation of high  $\text{K}^+$ -induced contractions. Procedure as for *a* except that taenia were contracted in PSS in which all  $\text{Na}^+$  was replaced by  $\text{K}^+$ . Relaxants were added 3 min after  $t_0$ .

The evidence also suggests that contraction is at least partially regulated by intracellular  $\text{Ca}^{2+}$  stores. Although the ouabain-induced myofilament activation seems to be controlled primarily by these stores, the high  $\text{K}^+$  depolarisation produces activation that depends largely on extracellular  $\text{Ca}^{2+}$ . Finally, note should be made of recent evidence<sup>13</sup> that cyclic AMP may effect relaxation by inactivating myosin light chain kinase, thus reducing tension at the myofilament level. The data presented here are equally consistent with this model and that of stimulated sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase.

Either or both of these mechanisms may be operative in taenia coli during  $\beta$ -mediated relaxation but our evidence rules out net efflux of  $\text{Ca}^{2+}$ , specifically that by a  $\text{Na}^+-\text{Ca}^{2+}$  exchange carrier.

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## Extracellular but not intracellular application of peptide hormones activates pancreatic acinar cells

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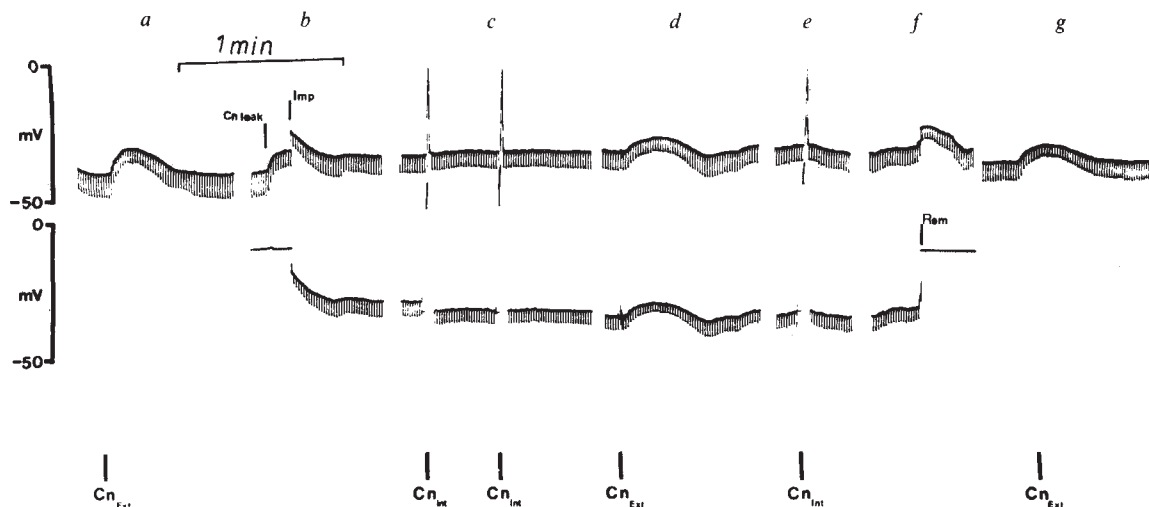
Peptide hormones, like neurotransmitters, are traditionally thought to activate cells by interacting with receptor sites accessible only from the extracellular space<sup>1-3</sup>. However, there is no available evidence that establishes whether intracellular injections of peptide secretagogues can or cannot initiate cell activation. In view of recent demonstrations that peptide hormones can penetrate the intracellular space in some tissues<sup>1,4</sup> and the reports that intracellular injections of the neurotransmitter, dopamine, into acinar cells of cockroach salivary gland cause stimulation<sup>5</sup> it seems of fundamental importance to test directly whether introduction of peptide secretagogues inside acinar cells of mammalian exocrine tissue can induce cell activation without first interacting with the outer surface of the external cell membrane. The data presented here show that injections of the secretagogue peptides caerulein and bombesin-nonapeptide (bombesin-NP) into pancreatic acinar cells fail to evoke the characteristic potential and conductance changes that are observed following extracellular applications of these peptides.

The experiments were carried out on segments of mouse pancreas superfused with a physiological saline solution equilibrated with 95% O<sub>2</sub>, 5% CO<sub>2</sub> at 37 °C. Membrane potentials and resistance from two electrically coupled acinar cells were measured with two microelectrodes and application of caerulein and bombesin-NP from microelectrodes using ionophoresis was

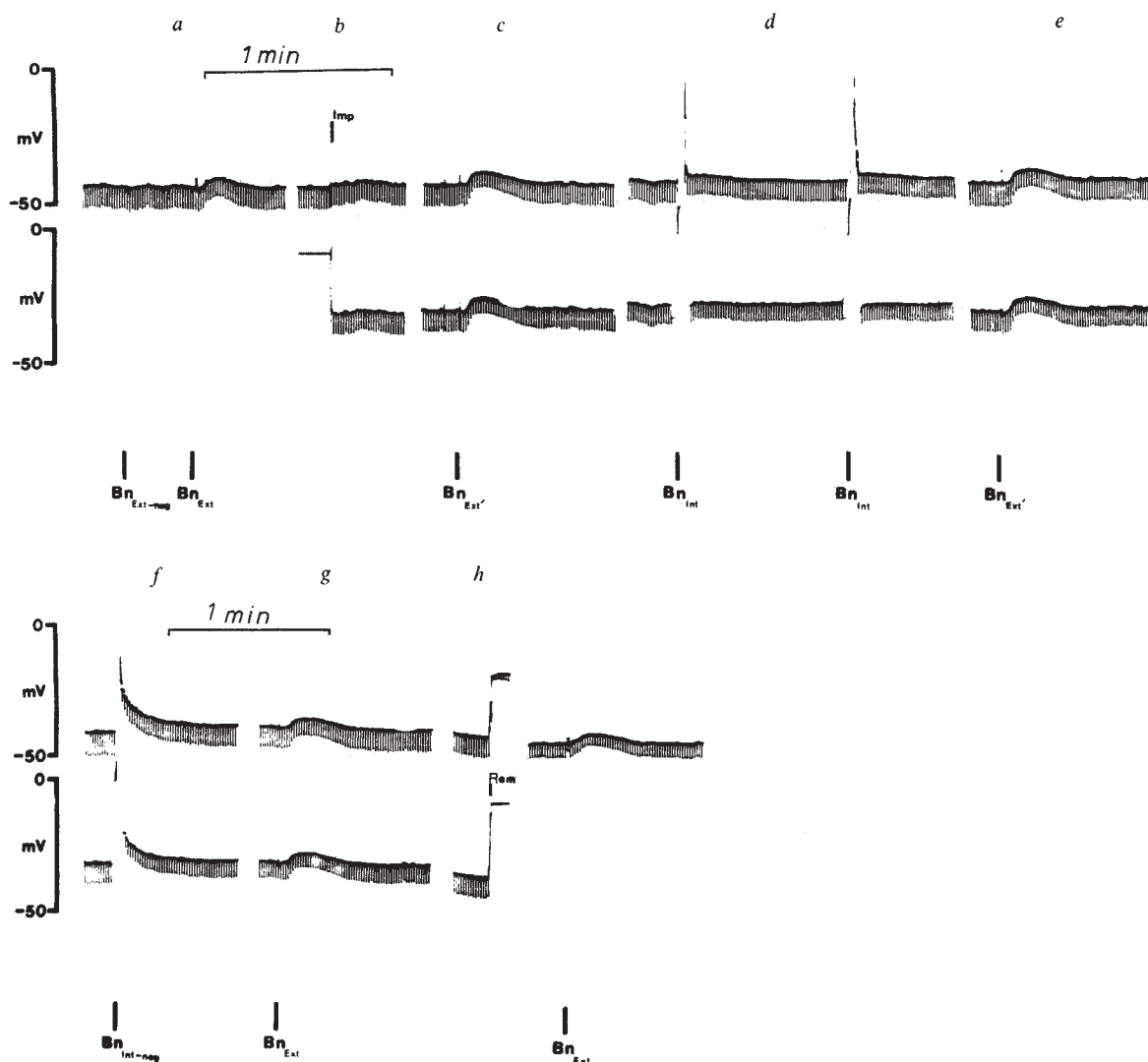
carried out as previously described<sup>6,7</sup>. One intracellular micro-electrode was filled with 3 M KCl and the other with 30  $\mu$ M caerulein (diethylamine salt) dissolved in 0.9% NaCl (pH 4) or 100  $\mu$ M bombesin-nonapeptide hydrochloride in 0.9% NaCl (pH 4) (Farmitalia). The extracellular micropipette was filled with the same peptide solution as that used for the intracellular electrode.

The effects of caerulein ionophoresis are shown in Fig. 1. Extracellular application evoked the characteristic cell depolarisation and reduction in input resistance from the recording cell. In contrast, after the caerulein electrode had been inserted into a communicating acinar cell, intracellular ionophoresis had no effect on the electrical properties of either of the two impaled cells, while extracellular caerulein application from another electrode still evoked the characteristic response. After withdrawal of the tip of the intracellular caerulein electrode to the extracellular space, the electrode was still able to deliver caerulein and thereby effect the usual electrical response from the cell that remained impaled with the KCl electrode. From the 32 intracellular ejections of caerulein that were carried out on 14 acinar cells in pancreatic tissue from five mice, no response from the cells was obtained. Of special interest in Fig. 1 is the effect of spontaneous leakage of caerulein from the caerulein electrode causing depolarisation of the recording cell<sup>7</sup>. This was abolished once the caerulein electrode had been impaled into an acinar cell, allowing both cells to repolarise.

Figure 2 demonstrates that intracellular ionophoresis of bombesin-NP has no effect on the electrical properties of the acinar cell membrane in conditions where extracellular bombesin-NP applications evoked clear membrane resistance and potential changes. Application of a negative current through an extracellular bombesin electrode does not release bombesin or cause stimulation, but application of a positive ejection current does achieve this. The membrane depolarisation effect observed after applying a negative, hyperpolarising



**Fig. 1** Effect of extracellular and intracellular ejection of caerulein, applied to pancreatic acinar cells, on the membrane potential and input resistance. Panels *a*–*g* are consecutive traces. In *a* the cell membrane potential was measured from an intracellular KCl microelectrode through which rectangular hyperpolarising current pulses ( $2 \times 10^{-9}$  A, 100 ms duration) were repeatedly injected to obtain cell input resistance. At Cn<sub>Ext</sub> a positive ionophoretic current (100 nA, 500 ms duration) was applied to a caerulein-containing electrode (Cn-electrode) whose tip was positioned near to the surface of the impaled cell. In *b* the Cn-electrode, previously used for extracellular ionophoresis, was impaled at 'Imp' into an acinar cell that was in electrical communication with the cell impaled with the KCl electrode. 'Cn-leak' indicates the depolarisation of the recording cell arising from caerulein leakage from the approaching tip of the caerulein electrode, before impalement. In *c* and *e* at places marked 'Cn<sub>Int</sub>' intracellular ionophoresis of caerulein was carried out. At *d* a second caerulein electrode was positioned near to the two indwelling electrodes and used for extracellular caerulein ionophoresis at 'Cn<sub>Ext</sub>'. In *f* the intracellular caerulein electrode was removed from the acinar cell at the place marked 'Rem'. In *g* after time was allowed for the first impaled cell to recover from disturbance caused by removal of the caerulein electrode, the same caerulein electrode was then used for extracellular ionophoresis at 'Cn<sub>Ext</sub>'. A positive injection current of 100 nA lasting 500 ms was used throughout the experiment for caerulein ionophoresis. Time intervals between panels *a* and *b*, 20 s; *b* and *c*, 40 s; *c* and *d*, 6 min; *d* and *e*, 60 s; *f* and *g*, 20 min.



**Fig. 2** Effect of extra- and intracellular ionophoresis of bombesin on pancreatic acinar cells. At 'Bn<sub>Ext-neg</sub>', a negative ejection current was applied to a bombesin-containing electrode, near to the surface of the impaled cell. A second but positive current was then applied at 'Bn<sub>Ext</sub>'. In *b* the Bn-electrode was inserted into an acinar cell coupled with the first recording cell. In *c* and *e* another extracellular bombesin electrode was placed near to the coupled cells and at 'Bn<sub>Ext</sub>' an ionophoretic current was then applied. In *d* at places marked 'Bn<sub>Int</sub>' intracellular ionophoresis of bombesin was carried out. In *f* at 'Bn<sub>Int-neg</sub>' the effects of passing a hyperpolarising current through the intracellular bombesin electrode are shown. *h*, Bn electrode removed at 'Rem'. *i*, At 'Bn<sub>Ext</sub>' bombesin was ionophoresed extracellularly on to the remaining impaled cell using the previously indwelling Bn electrode. Throughout the experiment an ejection current of 400 nA lasting 500 ms was used for bombesin-NP ionophoresis. Time intervals between panels *a* and *b*, 7 min; *b* and *c*, 2 min; *c* and *d*, 7 min; *d* and *e*, 3 min; *e* and *f*, 7 min; *f* and *g*, 50 s; *g* and *h*, 13 min; *h* and *i*, 7 min.

current through the intracellular bombesin electrode (Fig. 2*f*) is not, therefore, due to bombesin-induced effects, but results from breakdown in the dielectric properties of the membrane, usually associated with intracellular injection of large hyperpolarising currents<sup>8</sup>. Of the 37 intracellular injections of bombesin into 11 acinar cells obtained from four mice, 30 showed no effect on membrane potential and resistance, 5 results showed a small effect on membrane resistance but no depolarisation, and 2 results gave a small but definite depolarisation and resistance reduction when compared to the bombesin-evoked responses obtained with extracellular application using the same electrode. The exceptional intracellular bombesin depolarising effects were only observed in cells where the resting membrane potentials were extremely low ( $< -23$  mV). These effects are likely to have arisen from seepage of bombesin out of the acinar cell due to incomplete resealing of the cell membrane around the impaling electrode. Evidence for the leakage of

intracellularly applied calcium out of injected cells with very low membrane potentials, has previously been given<sup>9</sup>.

The experiments presented here are the first to monitor the electrical response of target cells during intracellular application of peptide hormones. The results provide evidence for the hypothesis that peptides like caerulein and bombesin, as also found for the neurotransmitter acetylcholine<sup>2,3</sup>, attach to receptors on the outer surface of cell membranes as a prerequisite to stimulation. Specific binding of radiolabelled bombesin to the plasma membrane of pancreatic acinar cells has recently been demonstrated<sup>10</sup>.

There seems to be a discrepancy between the results reported here and the study of House *et al.*<sup>5</sup> showing potential changes in cockroach salivary gland cells, with intracellular dopamine application. The apparent intracellular dopamine effects, however, as the authors suggested<sup>5</sup>, may have been due to leakage of injected transmitter into the extracellular compartment.



Having established that, in order to achieve cell activation, the pancreatic acinar secretagogues must first attach to receptors on the extracellular side of the plasma membrane, there seems to be a need for an intracellular mediator. Intracellular  $\text{Ca}^{2+}$  application has been shown to mimic the electrical effects induced by outside application of pancreatic secretagogues<sup>8,9</sup> and therefore seems the most likely intracellular messenger. An alternative but more complicated theory is that a further mediation step may occur and take the form of a peptide hormone/receptor complex undergoing cell internalisation. To test this possibility studies should be carried out in the pancreas.

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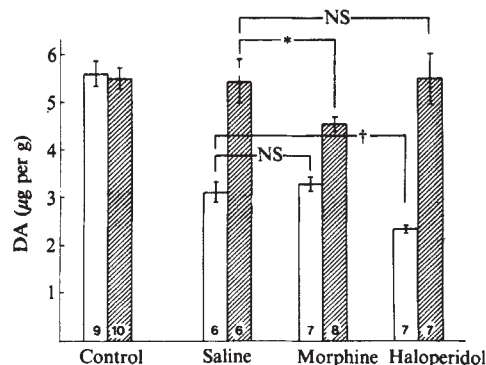
## Morphine-induced striatal dopamine efflux depends on the activity of nigrostriatal dopamine neurones

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It is clear that morphine markedly increases the turnover of dopamine (DA) in the rat striatum<sup>1–3</sup>, although the nature of this increase remains obscure. The early suggestion, that morphine may block postsynaptic DA receptors in a similar manner to haloperidol<sup>4</sup>, has since been refuted<sup>5,6</sup>. The effect of morphine on dopaminergic neurones seems to be presynaptic, but whether it stimulates<sup>7,8</sup> or inhibits<sup>2,5</sup> the functional activity of the dopaminergic neurones is unclear. We recently reported that morphine seems to have no effect on the functional activity of the nigrostriatal dopaminergic neurones, using the decline in DA after administration of  $\alpha$ -methyl-*p*-tyrosine ( $\alpha$ MT) as a measure of DA efflux<sup>9,10</sup>. However, the substantia nigra and striatum are extremely rich in opiate receptors<sup>11,12</sup>, and a considerable number of the striatal opiate receptors seem to be localised on dopaminergic nerve endings<sup>13</sup>; it would therefore be rather surprising if stimulation of these receptors by morphine did not alter the functional activity of the nigrostriatal dopaminergic neurones. To investigate this apparent discrepancy, we analysed the effect of morphine on DA efflux in conditions of altered impulse flow in the nigrostriatal dopaminergic neurones. The present results show that the administration of morphine results in increased efflux of DA from neurones with a lowered firing rate, but not from neurones with a normal or increased firing rate. Thus, morphine may be a modulator of the nigrostriatal dopaminergic neurones in that it counteracts decreasing dopaminergic activity, but has no effect when the activity is normal or increased.

Male albino Wistar rats were treated with  $\alpha$ MT methyl-ester.HCl (254 mg per kg, intraperitoneally (i.p.)) half an hour before further treatment, which was aimed at altering the activity of the nigrostriatal neurones. These various treatments consisted of unilateral electrolytic lesion of the substantia nigra,



**Fig. 1** Effect of morphine and haloperidol on the loss of striatal dopamine after  $\alpha$ MT treatment in rats with unilateral lesion of the mesencephalic dopaminergic cell bodies. Male albino Wistar rats weighing 195–205 g were treated according to the time schedule given below. Unilateral electrolytic lesions of the right mesencephalic dopaminergic cell bodies were made stereotactically by means of an iron electrode, 1 mm thick, coated with epoxy varnish except for 0.5 mm at the tip. A current of 2 mA was passed through the electrode for 3 min. The coordinates of the electrode were 6.6 mm caudal, 2.0 mm lateral and 7.7 mm ventral from the bregma. Midbrains were removed at the end of the experiment, fixed in formalin and examined histologically for electrode placements. All rats were injected with  $\alpha$ MT 25 min before the lesion was made. Seventeen minutes after lesioning, the control rats were decapitated, while the other rats received morphine, haloperidol or saline injection. Most animals, at the moment of drug injection, had recovered from the light ether anaesthesia applied before lesioning. Rats were decapitated 2 h after injection of morphine, haloperidol or saline. Striata were dissected out according to Gispén *et al.*<sup>14</sup> and DA was extracted and assayed as reported elsewhere<sup>10</sup>.

### Time schedule of treatments (min)

| Treatment group | 0           | 25     | 42           | 162          |
|-----------------|-------------|--------|--------------|--------------|
| Control         | $\alpha$ MT | Lesion | Decapitation | —            |
| Saline          | $\alpha$ MT | Lesion | Saline       | Decapitation |
| Morphine        | $\alpha$ MT | Lesion | Morphine     | Decapitation |
| Haloperidol     | $\alpha$ MT | Lesion | Haloperidol  | Decapitation |

$\alpha$ MT: 254 mg per kg; morphine: 20 mg per kg; haloperidol: 1 mg per kg. All injections were made i.p. in a volume of 5 ml per kg. Drugs were dissolved in 0.9% NaCl. Statistics: Duncan's new multiple range test<sup>22</sup>. Values represent mean  $\pm$  s.e.m.; number of observations are indicated at the base of the columns. Mean wet tissue weight: 46.1 mg  $\pm$  5.3. □, Control; ▨, lesion. \*  $P < 0.05$ ; †  $P < 0.01$ ; NS, not significant.

apomorphine or haloperidol administration. Morphine (20 mg per kg, i.p.) was administered 17 min after lesion or together with apomorphine or haloperidol. Two hours after morphine administration the rats were decapitated and the striata were dissected out as described by Gispén *et al.*<sup>14</sup>; DA was assayed fluorimetrically as reported earlier<sup>10</sup>.

Figure 1 shows that lesion of the mesencephalic dopaminergic cell bodies entirely prevented the loss of DA from the ipsilateral striatum. Injection of morphine resulted in loss of DA on the lesioned side but not on the side contralateral to the lesion, when compared with saline-treated rats. Haloperidol injection, on the other hand, resulted in increased loss of DA on the unlesioned side, but was without effect on the lesioned side. Thus, morphine seems to produce an efflux of DA from inactive neurones in the striatum but not from normally firing neurones. As the opposite is true for haloperidol, we conclude that the observed effect of morphine is not mediated by blockade of the DA receptors, which are blocked by haloperidol. In the nucleus accumbens similar effects of morphine and haloperidol were observed (unpublished results).

We then evaluated the effect of morphine on the loss of DA in rats pretreated with apomorphine or haloperidol. Apomorphine reduces and haloperidol increases the firing rate of the nigral

DA neurones<sup>15</sup>. Treatment of the rats with apomorphine alone resulted in a reduction of ~50% in the loss of DA. In these apomorphine-treated rats, as in lesioned rats, administration of morphine resulted in increased DA loss. When the loss of DA was increased, however, by the administration of haloperidol, morphine had no effect (Table 1). Therefore, morphine administration apparently results in the efflux of DA in the striatum as a function of the impulse flow.

Table 2 shows that the effect of morphine was most pronounced when the impulse flow was entirely abolished, that it was less pronounced, but still significant, when the impulse flow was reduced by 50%, and that it was virtually absent at normal or increased impulse flow.

The present experiments allow conclusions to be drawn on the effect of morphine on DA release, if one assumes that the loss of DA after  $\alpha$ MT treatment reflects DA release. During the first 30 min after  $\alpha$ MT administration DA biosynthesis is not completely inhibited<sup>9</sup> and DA-releasing metabolites of  $\alpha$ MT may be formed<sup>16</sup>. This time period is not suitable for studying DA release, therefore, but between 0.5 and 2.5 h after  $\alpha$ MT administration the loss of DA seems to be a reliable parameter for functional DA release<sup>17</sup>. However, drug-induced alterations in the loss of DA could result from intraneuronal events apart from functional DA release. It has been suggested that morphine increases the intraneuronal metabolism of DA by displacing DA from storage sites to sites of metabolism<sup>2</sup>. However, it is unlikely that the present results could be explained by such a mechanism. Although morphine administration to apomorphine-treated rats resulted in increased loss of DA (Table 1), we have shown that in the same experimental conditions morphine has no effect on the concentrations of the DA metabolites dihydroxyphenylacetic acid (DOPAC) and

**Table 1** Effect of morphine after  $\alpha$ MT treatment on the loss of dopamine in the striatum of rats simultaneously treated with apomorphine or haloperidol

| a                                   | DA* ( $\mu$ g per g) |                     | % DA <sup>†</sup> |              |
|-------------------------------------|----------------------|---------------------|-------------------|--------------|
|                                     | Saline               | Apomorphine         | Saline            | Apomorphine  |
| $\alpha$ MT+<br>saline              | 2.1 $\pm$ 0.10 (5)   | 3.1 $\pm$ 0.14 (7)‡ | 46 $\pm$ 2.2      | 69 $\pm$ 3.1 |
| $\alpha$ MT+<br>morphine            | 1.9 $\pm$ 0.09 (6)   | 2.6 $\pm$ 0.07 (8)‡ | 41 $\pm$ 2.0      | 57 $\pm$ 1.6 |
| P                                   | NS                   | <0.005              |                   |              |
| b                                   | DA* ( $\mu$ g per g) |                     | % DA <sup>†</sup> |              |
|                                     | Saline               | Haloperidol         | Saline            | Haloperidol  |
| $\alpha$ MT+<br>saline              | 3.0 $\pm$ 0.08 (7)   | 1.8 $\pm$ 0.09 (8)‡ | 45 $\pm$ 1.2      | 27 $\pm$ 1.4 |
| $\alpha$ MT+<br>morphine            | 2.8 $\pm$ 0.14 (8)   | 1.8 $\pm$ 0.08 (7)‡ | 42 $\pm$ 2.1      | 27 $\pm$ 1.2 |
| P                                   | NS                   | NS                  |                   |              |
| c Time-schedule of treatments (min) |                      |                     |                   |              |
|                                     | 0                    | 30                  | 90                | 150          |
| $\alpha$ MT                         | Saline               | Saline              | Saline            | Decapitation |
| $\alpha$ MT                         | Morphine             | Saline              | Saline            | Decapitation |
| $\alpha$ MT                         | Apomorphine          | Apomorphine         | Apomorphine       | Decapitation |
| $\alpha$ MT                         | Apomorphine          | Apomorphine         | Apomorphine       | Decapitation |
|                                     | + morphine           |                     |                   |              |
| $\alpha$ MT                         | Haloperidol          | —                   | —                 | Decapitation |
| $\alpha$ MT                         | Haloperidol          | —                   | —                 | Decapitation |
|                                     | + morphine           |                     |                   |              |

Male Wistar rats weighing 100–135 g were treated according to the time schedule given in c. Separate experiments were carried out with apomorphine (a), 4 mg per kg as HCl salt, and haloperidol (b), 3 mg per kg. All drugs were dissolved in 0.9% NaCl. In the apomorphine experiment 0.2 mg ml<sup>-1</sup> ascorbic acid was added to this. Statistics: Student's *t*-test. Striata of one rat weighed 105  $\pm$  8.3 mg (n = 29) in a and 101  $\pm$  8.4 mg (n = 34) in b (mean  $\pm$  s.d.). For other experimental details see Fig. 1 legend.

\* Mean dopamine concentrations  $\pm$  s.e.m., number of experiments in parentheses.

<sup>†</sup> Values represent mean dopamine concentrations  $\pm$  s.e.m., expressed as % of control values in untreated rats. Dopamine concentrations in untreated rats (100%): a, 4.51  $\pm$  0.17  $\mu$ g per g (n = 3); b, 6.61  $\pm$  0.25  $\mu$ g per g (n = 4).

‡ Different from the appropriate saline group (P < 0.001).

**Table 2** Effect of morphine on dopamine efflux in the striatum as a function of the activity of striatal dopaminergic neurones

| Treatment to alter dopaminergic activity | Rate of dopamine loss |               | Effect of morphine (morphine – saline) |
|------------------------------------------|-----------------------|---------------|----------------------------------------|
|                                          | Saline                | Morphine      |                                        |
| Lesion                                   | 4 $\pm$ 14%           | 33 $\pm$ 9%   | +29%*                                  |
| Apomorphine                              | 48 $\pm$ 10%          | 68 $\pm$ 7%   | +20%*                                  |
| None                                     | 100 $\pm$ 9%          | 105 $\pm$ 11% | +5%                                    |
| Haloperidol                              | 162 $\pm$ 10%         | 162 $\pm$ 9%  | 0%                                     |

Rate constants of dopamine loss were calculated from the data presented in Fig. 1 and Table 1. The rate constants obtained from rats not treated to alter dopaminergic activity and not treated with morphine was set at 100%. The respective 100% values were: lesion experiment:  $k = 0.292 \pm 0.0394$  h<sup>-1</sup>; apomorphine experiment:  $k = 0.310 \pm 0.0291$  h<sup>-1</sup>; haloperidol experiment:  $k = 0.323 \pm 0.0184$  h<sup>-1</sup>. The rate of dopamine loss in the experimental groups was calculated as a percentage of the above control values.

\* Significant difference, see Fig. 1 and Table 1.

homovanillic acid (unpublished results). Additionally, DiChiara *et al.*<sup>18</sup> have shown that in mice not pretreated with  $\alpha$ MT, the rise in DOPAC concentrations observed after morphine administration is entirely abolished in apomorphine-treated animals.

We conclude, therefore, from the present experiments, that administration of morphine results in increased dopamine release in the rat striatum, when the activity of the nigrostriatal neurones is decreased. As the administration of morphine resulted in release of dopamine in the striatum after lesion of mesencephalic cell bodies (Fig. 1), this effect does not seem to be mediated by the substantia nigra. It seems more likely, therefore, that morphine exerts its releasing effects by a direct action on the dopaminergic axons, perhaps by means of presynaptic opiate receptors on striatal dopaminergic neurones<sup>13</sup>. Whether the reported stimulation of dopaminergic neurotransmission by morphine via the substantia nigra<sup>8,19</sup> has an underlying mechanism similar to the effect reported here, remains to be established. The similarity in the effects of morphine and endorphins on striatal dopaminergic systems<sup>20,21</sup> suggests that one function of the endogenous opioid systems may be to counteract decreases in the activity of the nigrostriatal dopaminergic system.

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## Correlation between benzodiazepine receptor occupation and anticonvulsant effects of diazepam

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The benzodiazepines are potent anticonvulsants for a wide variety of experimental and clinical seizure disorders<sup>1,2</sup>. The demonstration of saturable, high-affinity and stereospecific binding sites for the benzodiazepines in the mammalian central nervous system suggests the presence of pharmacological receptors mediating the anticonvulsant properties of these compounds<sup>3,4</sup>. The good correlation between the anticonvulsant potencies of a series of benzodiazepines and their ability to inhibit <sup>3</sup>H-diazepam binding *in vitro* further supports this hypothesis<sup>3,4</sup>, but evidence for a direct interaction between benzodiazepines and their receptors, and a subsequent inhibition of seizure activity (or elevation of seizure threshold) is lacking<sup>5</sup>. Recent reports from our laboratory<sup>6</sup> and others<sup>7</sup> have demonstrated the feasibility of labelling benzodiazepine receptors *in vivo* following parental administration of tritiated benzodiazepine. This technique permits one to study the relationship between the anticonvulsant activity of the benzodiazepines *in vivo* and the number of 'drug-occupied' receptors *in vitro*. We now report that there is an excellent correlation between benzodiazepine receptor occupancy by diazepam and protection against pentylenetetrazol-induced seizures. Furthermore, these results demonstrate that only a small fraction of benzodiazepine receptors need be occupied to produce a complete anticonvulsant effect.

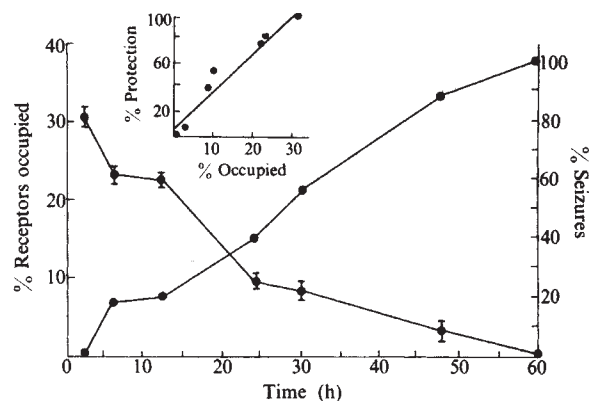
As previous studies have shown that the anticonvulsant activity of a single dose of diazepam decreases with time<sup>5</sup>, groups of 20 male -C3H/HEN mice (16–21 g) were injected with diazepam (4 mg per kg, intraperitoneally (i.p.), Hoffman-LaRoche) and the number of 'drug-occupied' benzodiazepine receptors was determined simultaneously with its anticonvulsant effects during a 72-h period. At each time point examined, half of the mice in each group were killed and both the apparent number of benzodiazepine receptors occupied by diazepam and the total concentration of benzodiazepine in forebrain<sup>8</sup> were determined. The remaining mice were challenged with pentylenetetrazol (80 mg per i.p., K and K Laboratories) to determine the per cent protected against seizures. Pentylenetetrazol-treated mice were counted as having had a seizure only if generalised tonic-clonic movements were observed within 15 min of injection. This dose of pentylenetetrazol produced seizures in >95% of vehicle-treated control mice.

In confirmation of previous reports<sup>5</sup>, the number of diazepam-treated mice having seizures in each group increased as a function of time following drug administration. Figure 1 shows the duration of the anticonvulsant effect following a single dose of diazepam. Greater than 90% of the mice were protected

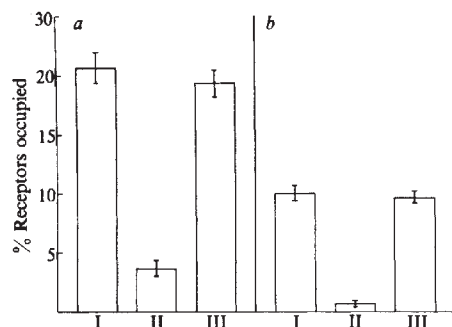
against seizures during the first 12 h after diazepam administration, and a significant protection was evident for as long as 48 h following treatment. However, at 60 and 72 h post-injection, all the mice exhibited seizure activity when challenged with pentylenetetrazol (Fig. 1 and unpublished observations).

The concentration of pharmacologically active 'non-membrane'-bound benzodiazepine in the brain decreased rapidly and was undetectable 24 h after diazepam administration, despite a significant anticonvulsant effect for an additional 24 h (Fig. 1). In contrast to the brain concentration of 'free' benzodiazepine, the number of benzodiazepine receptors occupied by diazepam (or its pharmacologically active metabolite, desmethyldiazepam) decreased slowly over time (Fig. 1). An excellent correlation ( $r=0.98$ ,  $P<0.01$ ) was observed between the number of drug-occupied receptors and protection against pentylenetetrazol-induced seizures (Fig. 1, inset). Occupation of only a small fraction (<30%) of the total number of forebrain benzodiazepine receptors was sufficient to elicit a full anticonvulsant effect.

To distinguish between drug-occupied receptors and receptor desensitisation which could occur due to prior drug-receptor interaction, it was necessary to demonstrate the presence of receptor-bound benzodiazepine in the diazepam-treated mice. As incubation of the benzodiazepine-receptor complex at 37 °C results in a rapid dissociation of drug from receptor<sup>9</sup>, crude synaptosomal membranes from diazepam-treated and vehicle-treated mice were incubated at 37 °C for 60 min followed by centrifugation at room temperature (12,000g for 15 min). The



**Fig. 1** Correlation between receptor occupation and anticonvulsant activity of diazepam. Mice were injected at time zero with diazepam (4 mg per kg, i.p.) and the number of drug-occupied benzodiazepine receptors was correlated with its anticonvulsant activity over the next 60 h. To determine the number of drug-occupied benzodiazepine receptors, mice were killed by decapitation and the crude synaptosomal fraction of forebrain (whole brain minus cerebellum and brainstem) was prepared by differential centrifugation<sup>3</sup>. The final synaptosomal pellet ( $P_2$ ) was resuspended in 40 volumes of ice-cold Tris-HCl buffer (0.05 M, pH 7.4) to a final protein concentration of  $\sim 1$  mg ml<sup>-1</sup>. The corresponding supernatant ( $S_2$ ) was frozen and stored at -20 °C for determining the free (unbound) concentration of benzodiazepine in brain<sup>8</sup>. Benzodiazepine receptors were measured in the resuspended  $P_2$  fraction by modification of previously described methods<sup>3</sup>. The per cent of receptors occupied by benzodiazepine in synaptosomal membranes was determined by the formula: % occupied =  $[(R_c - R_d)/B_{max}] \times 100$ , where  $R_c$  = <sup>3</sup>H-diazepam bound in vehicle-treated mice,  $R_d$  = <sup>3</sup>H-diazepam bound in diazepam-treated mice and  $B_{max}$  = maximum number of binding sites in vehicle-treated mice. The maximum number of binding sites in synaptosomal membranes from vehicle-treated mice was estimated by Scatchard analysis to be 1,213 fmol <sup>3</sup>H-diazepam bound per mg protein.  $R_c$  and  $R_d$  were determined in the presence of 4.4 nM <sup>3</sup>H-diazepam. At this concentration of <sup>3</sup>H-diazepam,  $R_c = 653 \pm 10$  fmol <sup>3</sup>H-diazepam bound per mg protein ( $n = 18$ ). The concentration of free or non-membrane-bound benzodiazepine was measured in the brain supernatants ( $S_2$ ) using a radioreceptor assay<sup>3</sup>.



**Fig. 2** Effects of *in vitro* incubation on receptor occupancy by benzodiazepines. Mice were injected with diazepam (4 mg per kg, i.p.) and synaptosomal membranes prepared 12 (a) or 24 (b) h later. Drug-occupied benzodiazepine receptors were determined as described in Fig. 1 legend. Group I: % receptor(s) occupied by benzodiazepines. Group II: % receptors occupied following incubation of membranes from group I (37 °C for 60 min), centrifugation (12,000g for 15 min) and reconstitution with supernatant from vehicle-treated mice. Group III: % receptor(s) occupied in membranes of vehicle-treated mice reconstituted with supernatant obtained from group II ( $n = 6$  mice per group).

resulting supernatants were carefully decanted and the supernatants from the diazepam-treated animals were reconstituted with synaptosomal membranes from the vehicle-treated animals; membranes from vehicle-treated mice were reconstituted with the supernatants from diazepam-treated animals. The reconstituted membranes from both groups were incubated for 30 min at 0–4 °C before incubation with  $^3\text{H}$ -diazepam.

Incubation (37 °C for 60 min) of crude synaptosomal membranes from mice treated with diazepam 12 h (Fig. 2a) and 24 h (Fig. 2b) earlier resulted in a marked decrease in the number of drug-occupied receptors. This decrease seems to be the result of a temperature-induced dissociation of benzodiazepine from the receptor, as exchanging supernatants from the diazepam-treated and vehicle-treated animals results in the detection of 'occupied receptor' in membranes from vehicle-treated mice. Furthermore, the decrease in the number of drug-occupied receptors in incubated diazepam-treated membranes is directly proportional to the increase observed in the control membranes following incubation with the 'diazepam-treated' supernatant (Fig. 2a, b). The presence of pharmacologically active benzodiazepine in the supernatant ( $S_2$ ) from the incubated diazepam-treated membranes was confirmed using a radioreceptor assay<sup>8</sup>.

These results demonstrate a close association between the number of drug-occupied benzodiazepine receptors and the anticonvulsant activity of diazepam. In contrast, the total concentration of 'free' benzodiazepine in brain is poorly correlated with its anticonvulsant effects. Only a small fraction (25–30%) of the total number of benzodiazepine receptors need be occupied to manifest a complete anticonvulsant effect. This observation could indicate the presence of 'spare receptors' similar to that reported for various peripheral hormonal and neurotransmitter systems<sup>10</sup>. Alternatively, a specific population of benzodiazepine receptors may be involved in the anticonvulsant effects of diazepam, and the correlation obtained reflects preferential binding of diazepam to these receptors<sup>11</sup>. In addition to providing evidence for the importance of the benzodiazepine receptor in mediating the anticonvulsant effects of the benzodiazepines, these findings represent the first direct correlation between the occupation of a central receptor site and the clinical effects of a drug. Furthermore, they suggest that the benzodiazepine receptor and its presumed endogenous ligand(s)<sup>12</sup> may normally be involved in preventing the discharge or spread of seizure activity within the CNS.

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## Glycyrrhizic acid inhibits virus growth and inactivates virus particles

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Screening investigations in antiviral action of plant extracts have revealed that a component of *Glycyrrhiza glabra* roots, found to be glycyrrhizic acid, is active against viruses. We report here that this drug inhibits growth and cytopathology of several unrelated DNA and RNA viruses, while not affecting cell activity and ability to replicate. In addition, glycyrrhizic acid inactivates herpes simplex virus particles irreversibly.

The effects of glycyrrhizic acid (ammonium salt, Fluka, Buchs) on growth of vaccinia, herpes simplex type 1, Newcastle disease, vesicular stomatitis (Indiana type) and polio type 1 viruses (all provided by NIH) in cultures of human aneuploid HEP2 cells (ATCC, Rockville) where studied. Twenty-four-hour-old cell monolayers ( $10^7$  cells per sample) were infected with 5 infectious units per cell of each virus at 20 °C for 1 h, washed 3 times in Hank's balanced saline solution (BSS) and incubated at 37 °C for 18 h in Eagle's minimum essential medium (MEM) supplemented with 2% calf serum (pH 7.4). Infectious virus yield was determined by the Dulbecco and Vogt technique<sup>1</sup>, slightly modified for vaccinia and herpes simplex type 1 viruses<sup>2</sup>. Cytopathic effects were evidenced by light microscope observation of Giemsa-stained cells and by measuring spectrophotometrically at 530 nm the amount of neutral red incorporated by cell cultures ( $100 \mu\text{g ml}^{-1}$ , 1-h pulses in a drug-free medium) after solubilisation in 1% sodium deoxycholate in BSS (pH 7.4)<sup>3</sup>.

As shown in Table 1, addition of 8 mM glycyrrhizic acid to infected cell cultures soon after incubation at 37 °C completely inhibits both growth and cytopathic effects of vaccinia, herpes simplex type 1, Newcastle disease and vesicular stomatitis viruses. Protection of cell cultures from viral damage is so effective that, in spite of the total infection produced by the virus input used, differences can hardly be found at microscopic observation levels between drug-treated infected cells and uninfected controls. Later treatment (3 h post-infection) of cell cultures with 8 mM glycyrrhizic acid still suppresses virus replication and stops the progress of the cytopathic effect. Glycyrrhizic acid is also active on virus growth at 4 mM and at 2 mM, but not at 1 mM (data not shown). On the other hand, glycyrrhizic acid is without effect on poliovirus type 1.

Glycyrrhizic acid treatments that were inhibitory to virus growth were tolerated by cell cultures.

Uninfected cells incubated at 37 °C for 36 h in the presence of 8 mM glycyrrhizic acid remained unaltered both morphologically (evaluated as above) and in their ability to incorporate



**Table 1** Effect of glycyrrhizic acid on virus growth and cytopathology

| Virus strain<br>input (5 i.u. per cell) | Virus growth and cell damage after 18 h at 37 °C in: |     |    |                                                  |     |     |                           |     |    |
|-----------------------------------------|------------------------------------------------------|-----|----|--------------------------------------------------|-----|-----|---------------------------|-----|----|
|                                         | Drug-free medium                                     |     |    | Medium supplemented with 8 mM glycyrrhizic acid: |     |     |                           |     |    |
|                                         | i.u.                                                 | CPE | NR | Immediately after infection<br>i.u.              | CPE | NR  | 3 h postinfection<br>i.u. | CPE | NR |
| Vaccinia                                | $6.8 \times 10^7$                                    | +++ | 45 | $2 \times 10^4$                                  | —   | 99  | $3 \times 10^4$           | +++ | 86 |
| Herpes simplex type 1                   | $3.4 \times 10^7$                                    | +++ | 32 | $<10^4$                                          | —   | 97  | $<10^4$                   | +++ | 84 |
| Newcastle disease                       | $1.3 \times 10^7$                                    | +++ | <5 | $6 \times 10^4$                                  | —   | 102 | $9 \times 10^4$           | +++ | 82 |
| Vesicular stomatitis                    | $1.2 \times 10^8$                                    | +++ | <5 | $6 \times 10^4$                                  | —   | 96  | $1.3 \times 10^5$         | +++ | 78 |
| Polio type 1                            | $7 \times 10^7$                                      | +++ | <5 | $4.6 \times 10^7$                                | +++ | <5  | $5.2 \times 10^7$         | +++ | <5 |

i.u., Infectious units; CPE, cytopathic effect; +++, complete CPE, —, absence of CPE, evaluated microscopically; NR, neutral red uptake in % of untreated uninfected controls.

**Table 2** Effects of glycyrrhizic acid on uninfected cell cultures

| Glycyrrhizic acid in medium | $^{14}\text{C}$ amino acid uptake*<br>(c.p.m.) | No. of cells per ml of medium†                              |
|-----------------------------|------------------------------------------------|-------------------------------------------------------------|
| —                           | 76.705 (74.534–80.212)                         | $7.1 \times 10^5$ ( $6.2 \times 10^5$ – $7.9 \times 10^5$ ) |
| 8 mM                        | 73.952 (71.528–77.334)                         | $5.2 \times 10^5$ ( $4.2 \times 10^5$ – $6.4 \times 10^5$ ) |

Figures are mean values of six determinations. The range of values is given in parentheses.

\*  $^{14}\text{C}$  amino acid uptake ( $0.3 \mu\text{Ci ml}^{-1}$ , 1-h pulses) was measured after 35 h at 37 °C in Eagle's MEM 2% serum.

† Initial inoculum,  $2.5 \times 10^5$  cells  $\text{ml}^{-1}$ . Count made after 36 h at 37 °C in Eagle's MEM 7% serum.

amino acids in acid-insoluble form<sup>4</sup>, as determined by 1-h pulses of  $^{14}\text{C}$ -protein hydrolysate ( $40 \text{ mCi mmol}^{-1}$ ,  $0.3 \mu\text{Ci ml}^{-1}$ , Amersham) (Table 2). Addition of 8 mM glycyrrhizic acid to 4-h-old cell cultures incubated in Eagle's MEM 7% calf serum had virtually no effect on cell growth. Thoma chamber counts of cells suspended by trypsin (0.25%, Difco) revealed a 28% decrease in cell growth in the respect of untreated controls, after 36 h at 37 °C.

Taking into account that in antiviral tests glycyrrhizic acid was allowed to act on infected cultures for 18 h (the time required for growth of all viruses in untreated controls), these data indicate that the antiviral effect of this drug is not mediated by damaging the cells.

Apart from inhibiting growth of several viruses, glycyrrhizic acid also produces irreversible inactivation of herpes simplex virus type 1. Suspensions of this virus suffer a loss of infectivity of  $10^5$  when incubated at 37 °C with 8 mM glycyrrhizic acid for only 15 min. Similar treatments do not inactivate the other viruses (at least irreversibly), even if prolonged for 3h.

**Table 3** Inactivating effect of glycyrrhizic acid on virus particles

| Virus strain          | Incubation mixture at 37 °C | i.u. Evidenced*   |
|-----------------------|-----------------------------|-------------------|
| Vaccinia              | in MEM                      | $5.3 \times 10^7$ |
|                       | in 8 mM GA                  | $5.6 \times 10^7$ |
| Herpes simplex type 1 | in MEM                      | $1.2 \times 10^7$ |
|                       | in 8 mM GA                  | $1.3 \times 10^2$ |
| Newcastle disease     | in MEM                      | $3.9 \times 10^7$ |
|                       | in 8 mM GA                  | $4.2 \times 10^7$ |
| Vesicular stomatitis  | in MEM                      | $4.3 \times 10^7$ |
|                       | in 8 mM GA                  | $3.1 \times 10^7$ |
| Polio type 1          | in MEM                      | $6.1 \times 10^7$ |
|                       | in 8 mM GA                  | $3.9 \times 10^7$ |

\* By the Dulbecco and Vogt method<sup>1</sup> in incubation mixtures diluted in drug-free MEM.

$5 \times 10^7$  i.u. of each virus was incubated for 180 min, except in the case of herpes simplex type I in 8 mM GA, which was for 15 min. GA, glycyrrhizic acid.

Data from current research had led us to hypothesise that glycyrrhizic acid interacts with sensitive virus proteins both at the virionic stage and later on, when these proteins are synthesised in host cells. This is, however, a working hypothesis that has yet to be verified experimentally.

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## Ca<sup>2+</sup>-induced fusion of phospholipid vesicles monitored by mixing of aqueous contents

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Ca<sup>2+</sup> has a central role in various cellular phenomena involving membrane fusion<sup>1–3</sup>. However, little is known about the mechanisms involved. Model membrane systems such as phospholipid vesicles have been used extensively to study the mechanism of membrane fusion at the molecular level<sup>4</sup>. For example, phosphatidylserine (PS) vesicles have been shown to undergo massive aggregation and structural rearrangements on addition of Ca<sup>2+</sup>, with eventual formation of large cochleate structures<sup>5–8</sup>. Although these structures do not retain appreciable internal volume, their formation has been proposed to result from fusion of the initial vesicles<sup>5–8</sup>. The significance of the PS–Ca<sup>2+</sup> system as a model for biological membrane fusion has been questioned recently by Ginsberg<sup>9</sup>. Based on the observation that divalent cations induce the release of contents from PS vesicles but fail to bring about the uptake of a marker from the medium, he proposes that the vesicles are ruptured completely during interaction with divalent cations and reassemble subsequently to form large non-vesicular structures. The present study demonstrates that the question raised by Ginsberg<sup>9</sup> is not particularly relevant to the phenomenon concerned, and that his experimental observations do not allow the exclusive conclusion that Ca<sup>2+</sup> induces lysis of PS vesicles rather than fusion.

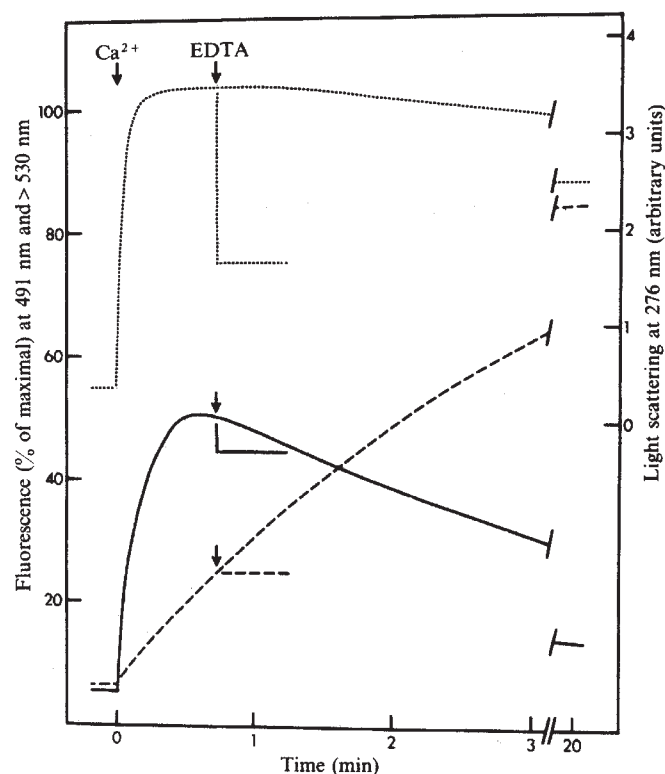
With respect to the question of 'lysis or fusion', in general, membrane fusion is not necessarily a completely non-leaky process. Therefore, release of vesicle contents induced by

divalent cations does not preclude a fusion event being involved. However, the question of whether the release of contents depends on mutual interaction between the vesicles is crucial. Our recent observation that  $\text{Ca}^{2+}$ -induced leakage of PS vesicle contents is approximately second-order with respect to vesicle concentration<sup>8</sup> indicates that vesicle-vesicle interaction is a prerequisite for the observed release and suggests that leakage from separate PS vesicles does not occur. The observation by Ginsberg that  $\text{Ca}^{2+}$  fails to bring about the uptake or entrapment of markers from the external medium into the vesicles<sup>9</sup> can be completely reconciled with earlier results<sup>5,6</sup> regarding the formation of large cochleate structures from PS vesicles by interaction with  $\text{Ca}^{2+}$ . These cochleates have been shown to result from the eventual collapse of the vesicles and, therefore, do not retain any encapsulated volume<sup>7,8</sup>. This is indicated by the very short lamellar distance of 53 Å in the PS- $\text{Ca}^{2+}$  membrane system, determined by X-ray diffraction<sup>7</sup>, and the absence of any appreciable amounts of water between the bilayers in the final complex<sup>8</sup>. Consequently, it is highly unlikely that solutes from the medium would be taken up and retained in the structure during a 1-h incubation. However, the final formation of such a tightly packed PS- $\text{Ca}^{2+}$  membrane complex does not necessarily lead to the conclusion that the sequence of events involved is one of lysis of separate vesicles with subsequent reassembly into non-vesicular structures, as proposed by Ginsberg<sup>9</sup>. As the author admits, fusion might occur during the initial stages of the interaction between vesicles. However, the design of his experiments did not permit investigation of the early events in this process.

To be able to follow the kinetics of vesicle fusion phenomena, we have developed an assay which meets the rigorous criterion of mixing of vesicle contents. The method is based on the interaction between terbium ( $\text{Tb}^{3+}$ ) ions and dipicolinic acid (DPA)<sup>10-12</sup>. Formation of the  $\text{Tb}(\text{DPA})_3^{3-}$  complex produces a  $10^4$ -fold enhancement of the Tb fluorescence<sup>11,12</sup>. The design of the assay involves encapsulation of  $\text{TbCl}_3$  in one population of vesicles and DPA in another.  $\text{TbCl}_3$  is encapsulated in the presence of a 10-fold molar excess of citrate as a weak chelator, which prevents interaction of  $\text{Tb}^{3+}$  with the negatively charged lipid. DPA is encapsulated at a 10-fold higher molar concentration than  $\text{TbCl}_3$ . On fusion of the two types of vesicle the  $\text{Tb}^{3+}$ -citrate complex is expected to dissociate in favour of the formation of the chelation complex between  $\text{Tb}^{3+}$  and dipicolinate, due to the very high affinity of the latter for  $\text{Tb}^{3+}$  (ref. 10). Release of vesicle contents is not registered as fusion because  $\text{Ca}^{2+}$  and EDTA, both present in the external medium, strongly interfere with the formation of the fluorescent  $\text{Tb}(\text{DPA})_3^{3-}$  complex through their interaction with DPA and  $\text{Tb}^{3+}$ , respectively. Introduction of 0.1 mM EDTA into a solution containing 1  $\mu\text{M}$   $\text{TbCl}_3$ , 10  $\mu\text{M}$  citrate and 10  $\mu\text{M}$  DPA in the buffer in which the fusion assay is done produces a 90% decrease of the fluorescence intensity. The additional presence of  $\text{Ca}^{2+}$  at a concentration of 1 mM or higher further reduces the fluorescence intensity to 1% of the maximal value. Moreover, during the fusion assay the quenching potencies of  $\text{Ca}^{2+}$  and EDTA will be even more pronounced, as the overall Tb, citrate and DPA concentrations are about seven-fold lower than those in the control experiment mentioned above. Consequently, only mixing of vesicle contents, separated from the outside medium, is registered as fusion. An additional advantage of this assay is that the formation of the  $\text{Tb}(\text{DPA})_3^{3-}$  complex is extremely fast and thus allows an accurate kinetic analysis of the initial events during vesicle-vesicle interactions. A more extensive account of the assay will be presented elsewhere.

Figure 1 shows the kinetics of mixing of vesicle contents induced by 1.5 mM  $\text{Ca}^{2+}$  in a 1:1 mixture of two PS vesicle populations containing Tb and DPA, incorporated in separate vesicles, as monitored by the increase of Tb fluorescence (solid line). The design of the fluorimeter permitted the simultaneous recording of the kinetics of vesicle aggregation, as measured by light scattering (dotted line). In addition, Fig. 1 shows the release of carboxyfluorescein (CF) from a similar vesicle preparation

induced by the same  $\text{Ca}^{2+}$  concentration (dashed line). It is apparent from these results that  $\text{Ca}^{2+}$  induces a very rapid mixing of the vesicle contents, which occurs on the same time scale as the initial aggregation. To exclude the possibility that the observed increase in fluorescence resulted from the formation of  $\text{Tb}(\text{DPA})_3^{3-}$  complexes in close proximity but outside the vesicles, possibly due to release to locally high concentrations,



**Fig. 1** Calcium-induced fusion of PS vesicles and release of vesicle contents. Unilamellar vesicles were prepared from bovine brain phosphatidylserine (PS) by sonication under nitrogen in a bath-type sonicator for 1 h at 20°C (ref. 6). Lipid concentration during sonication was 10  $\mu\text{mol ml}^{-1}$ . Thin-layer chromatographic analysis<sup>17</sup> of the lipid in vesicle preparations after sonication (with a load of 6  $\mu\text{g}$  of lipid phosphorus per spot on the thin-layer plate) showed no detectable amounts of degradation products such as lyso-PS and fatty acids. Vesicles were prepared in (1) 10 mM  $\text{TbCl}_3$ /100 mM sodium citrate/2.0 mM L-histidine/2.0 mM N-Tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES), pH 7.4; (2) 100 mM sodium dipicolinate (DPA)/2.0 mM L-histidine/2.0 mM TES, pH 7.4 or (3) 100 mM carboxyfluorescein (CF)/2.0 mM L-histidine/2.0 mM TES, pH 7.4, and separated from non-encapsulated material by gel filtration on Sephadex G-75. Elution buffer, 100 mM NaCl/2.0 mM L-histidine/2.0 mM TES/1.0 mM EDTA, pH 7.4. Phospholipid phosphorus was determined as described in ref. 18. Carboxyfluorescein was purified as described in ref. 13. All chemicals were of the highest purity available<sup>8</sup>. Fusion experiments were carried out in quartz fluorescence cells containing 1.0 ml of 100 mM NaCl/2.0 mM L-histidine/2.0 mM TES/0.1 mM EDTA, pH 7.4, at a total lipid concentration of 100  $\mu\text{M}$ . Tb- and DPA-containing vesicles were mixed in a 1:1 ratio, 50  $\mu\text{M}$  each. The solution was stirred with a magnetic stirring bar and the temperature was maintained at 25°C.  $\text{CaCl}_2$  was injected as a 100 mM solution to a final concentration of 1.5 mM. In separate experiments EDTA was injected as a 100 mM solution, 45 s after the addition of  $\text{CaCl}_2$ , to a final concentration of 5.0 mM. Fluorescence was measured in an SLM-4000 instrument (SLM Instruments) with a 'T'-design, permitting simultaneous recording of 90° fluorescence and 90° light scattering. Excitation of Tb fluorescence was at 276 nm, emission was detected at 491 nm (solid line), and light scattering was measured using a Corning 7-54 bandpass filter (dotted line). Excitation of CF fluorescence was at 460 nm and emission was measured at >530 nm using a Corning 3-68 cut-off filter (dashed line). The value of 100% Tb fluorescence was determined in the presence of 20  $\mu\text{M}$  DPA by releasing the contents from 50  $\mu\text{M}$  Tb-containing vesicles (freed from EDTA on Sephadex G-75) with 0.5% (w/v) sodium cholate. Maximal CF fluorescence was determined in the presence of 0.1% (v/v) Triton X-100. Fluorescence intensity is presented as a percentage of the maximal values. The light scattering is expressed in arbitrary units; note that the curve is shifted on the vertical scale.



we added excess EDTA to the mixture to terminate the fusion reaction once the maximum level of fluorescence had been attained. As shown in Fig. 1, the fluorescence intensity could be maintained at a high level, indicating a physical separation of the fluorescent complex from the external EDTA-containing medium. Although release of vesicle contents, as monitored by increasing CF fluorescence<sup>8,13</sup>, started immediately after  $\text{Ca}^{2+}$  addition, its rate was considerably lower than that of the Tb fluorescence increase. This indicates that fusion proceeds more rapidly than the release of vesicle contents. The Tb fluorescence reached its maximal level, corresponding to ~50% of the total amount of Tb present, within 45 s after the addition of  $\text{Ca}^{2+}$ . At that time, only 20–25% of the CF had been released. On addition of 2.0 mM  $\text{Ca}^{2+}$ , rather than 1.5 mM, the maximal level of Tb fluorescence approached 65% (not shown). These observations imply that multiple fusion events must occur, for otherwise, assuming no release at all, in a 1:1 mixture of the two types of vesicle only a maximum of 50% of the Tb can become associated with DPA. The eventual decrease of the Tb fluorescence (Fig. 1) is consistent with the release and dissociation of the  $\text{Tb}(\text{DPA})_3^{3-}$  complex in the medium. Addition of EDTA, 45 s after the addition of  $\text{Ca}^{2+}$  (Fig. 1) not only produces a constant high level of Tb fluorescence, but also stops CF release and induces a decrease in the light scattering to a level which is considerably higher than the level before  $\text{Ca}^{2+}$  addition, indicating increased vesicle size.

From the present results, we conclude that  $\text{Ca}^{2+}$  induces extensive fusion of PS unilamellar vesicles as monitored by mixing of aqueous contents. Leakage of vesicle contents, although remaining limited initially, does occur. Consequently,  $\text{Ca}^{2+}$  can slowly enter the vesicles where it can bind to the PS molecules of the interior monolayer, causing subsequent collapse of the fused vesicles and further release of contents. The assay system described here has made it possible to distinguish the initial fusion of PS vesicles from the release of vesicle contents. Studies with vesicles composed of various lipid mixtures are now in progress to investigate the possibility of producing vesicles which are capable of fusing but are more resistant to leakage than vesicles composed of pure PS. In any case, the present results and other recent reports<sup>8,14–16</sup> indicate that  $\text{Ca}^{2+}$ -induced vesicle fusion is quite relevant to biological membrane fusion phenomena, and could considerably enhance our understanding of the mechanism of such events at the molecular level.

$\text{TbCl}_3 \cdot 6\text{H}_2\text{O}$  was obtained from Alfa, dipicolinic acid from Sigma and carboxyfluorescein from Eastman Kodak. One of us (J.W.) is on leave from the University of Groningen, supported by the Netherlands Organisation for the Advancement of Pure Research (ZWO). This work was supported by grant GM 26369 from the NIH.

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## Differences in A and B forms of monoamine oxidase revealed by limited proteolysis and peptide mapping

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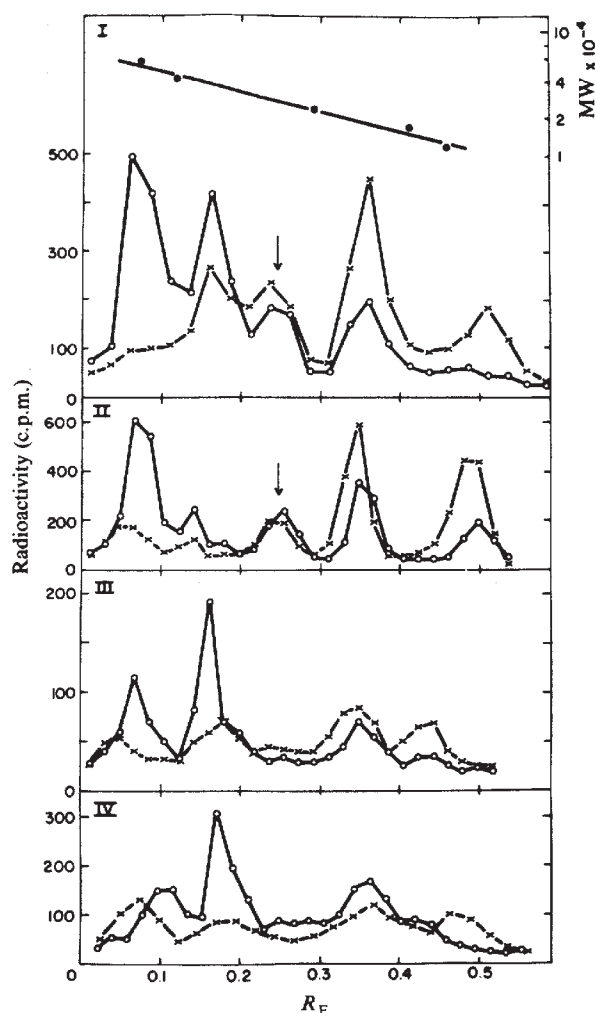
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Monoamine oxidase (MAO, EC 1.4.3.4) is a mitochondrial outer membrane enzyme which deaminates amine transmitters in the nervous system and biogenic amines throughout the body. At least two types of MAO activity have been identified which differ in their specificities for substrates and sensitivities to inhibitors<sup>1–3</sup>. Type A preferentially deaminates 5-hydroxytryptamine and noradrenaline and is more sensitive to inhibition by clorgyline. Type B deaminates phenylethylamine and benzylamine and is more sensitive to inhibition by deprenyl. Tissues from several species express MAO A alone, MAO B alone, or both types of MAO. Furthermore, the two types of activity follow different developmental sequences in certain tissues. Models to explain the molecular and genetic regulation of this enzyme<sup>4</sup> raise two questions: (1) are there different enzyme molecules associated with A and B types of activity, and (2) if there is only one enzyme molecule, how do differences in the microenvironment of this enzyme within the outer mitochondrial membrane produce two types of activity? Attempts to separate and purify the different types of activity have involved solubilisation and lipid extraction procedures that alter the kinetics of the enzyme and differentially affect the A and B types of activity<sup>5–8</sup>. Immunological studies<sup>9,10</sup> have produced equivocal results. None of these observations has elucidated the molecular basis of the differences between the types of MAO activity. Here, we have used the technique of proteolytic digestion and peptide mapping to demonstrate that there are distinct enzyme molecules associated with the A and B types of MAO activity. This finding implies that the A and B forms of MAO may be coded for by separate gene loci and represent evolutionarily related proteins which have diverged in function.

Pargyline, an irreversible inhibitor of both A and B types of MAO activity<sup>11–16</sup>, binds stoichiometrically to MAO, forming a stable adduct with the flavin residue<sup>16</sup>. This reaction is specific for MAO and does not occur with other purified flavoproteins<sup>17</sup>. Pargyline binding was used with limited proteolysis and one-dimensional peptide mapping, an effective technique for detecting differences and similarities in the amino acid sequences of proteins of similar molecular weights (MWs)<sup>18–20</sup>. The pattern of fragments obtained is highly reproducible and characteristic for a given substrate protein and proteolytic enzyme. As they are known to have similar MWs<sup>14,15</sup>, the structure of the A and B forms of MAO in rat hepatoma cells was investigated by site-specific proteolysis in the presence of SDS, followed by electrophoresis in SDS-polyacrylamide gels to identify peptide fragments.

Following binding of <sup>3</sup>H-pargyline to crude mitochondrial preparations, proteins were fully solubilised and denatured by heating in high concentrations of SDS in reducing conditions (see Fig. 1 legend), and electrophoretically separated on SDS-polyacrylamide gels. Figure 1 shows <sup>3</sup>H-pargyline-labelled peptide fragments detected when radiolabelled MAO was isolated and exposed to *Staphylococcus aureus* V8 protease and further SDS-polyacrylamide gel electrophoresis. The peptide maps in each panel are chosen to illustrate all the fragments obtained when identical samples of <sup>3</sup>H-pargyline-labelled MAO were incubated with five concentrations of protease. The first three panels display proteolytic fragments of MAO from rat

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Miles), stored at  $-20^{\circ}\text{C}$ , was thawed, and serial dilutions were prepared and added to the sample wells in amounts of 0, 0.005, 0.025, 0.1, 0.5, and 2.5  $\mu\text{g}$  per well. Standard MW marker proteins solubilised in loading solution (see above) were applied to the remaining sample well. The marker proteins used were catalase, 58,000 (Calbiochem); ovalbumin, 43,000; trypsin, 23,300; myoglobin, 17,200; cytochrome *c*, 12,400 (Sigma). Electrophoresis was carried out at a constant current of 45 mA, except for a 30-min period when current was turned off, after the tracer dye had neared the bottom of the stacking gel. Electrophoresis was continued until the bromophenol blue tracer dye in the marker lanes had moved  $\sim 10$  cm into the running gel. The gels were then stained and destained in  $< 1$  h, as above. Each lane was cut into approximately 50 2-mm slices and the distribution of radioactivity in the slices was quantitated as described above. A plot of the mobilities of the marker proteins against the log of their MWs was used to estimate the MWs of the radiolabelled peptide fragments. Panels I, II and III show peptides from line  $\text{MH}_1\text{C}_1$ : I, mitochondria were labelled with  $^3\text{H}$ -pargyline; II, mitochondria were incubated with clorgyline before  $^3\text{H}$ -pargyline labelling; III, mitochondria were incubated with deprenyl before  $^3\text{H}$ -pargyline labelling. Panel IV shows mitochondria from line C6, labelled with  $^3\text{H}$ -pargyline. Each panel shows peptide patterns from two of the five concentrations of protease tested. These patterns were chosen because they included all the peptide fragments observed at all protease concentrations. Results shown are from one of two or three similar experiments. Control samples of  $\text{MH}_1\text{C}_1$  incubated with clorgyline or deprenyl only after  $^3\text{H}$ -pargyline labelling yielded peptide maps essentially identical to that in panel I. In control samples not treated with protease, all radioactivity above background levels was found in the leftmost peak, corresponding to the MW of intact MAO—57,000.

**Fig. 1** Peptide maps of the A and B forms of MAO produced by limited proteolysis with *S. aureus* V8 protease and electrophoresis in 15% SDS-polyacrylamide gels. The migration relative to the tracer dye front ( $R_F$ ) is plotted on the abscissa, and the radioactivity of each slice and the MW are plotted on the left and right ordinates, respectively. The conditions and procedures used to grow and subculture the cell lines used were as described previously<sup>14</sup>, except that for subculturing, cells were resuspended in isotonic buffered saline containing no  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  and supplemented with 1 mM EDTA. Crude mitochondrial fractions were prepared from cultures collected at confluency as described elsewhere<sup>14</sup>, and stored at  $-20^{\circ}\text{C}$  or used immediately. To label MAO, mitochondrial preparations (400  $\mu\text{g}$  protein per sample) were incubated with 0.29 nmol [ $^3\text{H}$ -benzylmethyl]pargyline (6.86 Ci  $\text{mmol}^{-1}$ , NEN) in a final volume of 1.0 ml 50 mM Tris-HCl, pH 7.5, for 60 min at  $37^{\circ}\text{C}$  in a shaking water bath. Preparations in panel II were preincubated with 0.1  $\mu\text{M}$  clorgyline at  $37^{\circ}\text{C}$  for 30 min before addition of  $^3\text{H}$ -pargyline. Preparations in panel III were preincubated with 1.0  $\mu\text{M}$  deprenyl at  $37^{\circ}\text{C}$  for 15 min before addition of  $^3\text{H}$ -pargyline. To ensure that the drugs did not interfere with the proteolysis of MAO, some mitochondrial preparations were incubated with clorgyline or deprenyl only after the  $^3\text{H}$ -pargyline radiolabelling step. When all incubations were completed, the samples were centrifuged at 40,000g for 30 min at  $4^{\circ}\text{C}$ . The radiolabelled mitochondrial pellets were resuspended in 160  $\mu\text{l}$  of a loading solution containing 3% SDS, 0.15 M sucrose, 1%  $\beta$ -mercaptoethanol, 0.005% bromophenol blue, 3 mM EDTA and 0.0625 M Tris-HCl, pH 6.8, and proteins were solubilised by boiling for 5 min. Preparative polyacrylamide slab gel electrophoresis was carried out in a 7.5% acrylamide running gel ( $15 \times 10 \times 0.3$  cm) with a 3% stacking gel ( $15 \times 2 \times 0.3$  cm) both containing 0.1% SDS, using a modification<sup>23</sup> of the procedure of Maizel<sup>24</sup>. To locate the position of MAO in the preparative gel, a lane containing radiolabelled MAO and a lane containing standard MW marker proteins were sliced from the gel and stained and destained as described elsewhere<sup>18</sup>. The lane containing the mitochondrial sample was cut into 2-mm slices along a 2-cm section in the region of the 58,000 MW marker, catalase. The remainder of the preparative gel was stored, unstained, at  $-20^{\circ}\text{C}$ . The distribution of the radioactivity in the slices was determined quantitatively using a modification of the method of Horvitz<sup>25</sup> as recommended by NEN. Each slice was placed in a glass scintillation vial and 10 ml of a solution containing 5% Protosol and 7.5% Liquifluor (v/v, NEN) in toluene were added. Samples were incubated for 16 h at  $37^{\circ}\text{C}$  and radioactivity was determined by liquid scintillation spectrometry. A single peak of radioactivity was observed in the sliced region, indicating the precise location of MAO. The remainder of the preparative gel was then thawed, stained and destained in  $< 1$  h<sup>18</sup>, and cut to obtain nine gel slices, one  $9 \times 3 \times 3$ -mm slice per lane, which contained the radiolabelled MAO. The slices, and the sample wells of the proteolytic gels, were treated using a modification<sup>19</sup> of the method of Cleveland *et al.*<sup>18</sup>. The proteolytic gels were prepared as described<sup>18</sup> except that they were cast 3 mm thick with sample wells 2.5 cm deep and 1.0 cm wide. Three gel slices containing radiolabelled MAO were stacked at the bottom of each sample well. Six identical samples of three slices each were routinely loaded onto each gel. A concentrated solution of *S. aureus* V8 protease (36-900-1,

hepatoma line,  $\text{MH}_1\text{C}_1$ . Panel I shows peptide fragments derived when both the A and B forms of MAO were labelled. Panel II shows peptides exclusively from the B form, as binding of  $^3\text{H}$ -pargyline to the active site of type A MAO was blocked by preincubation of the mitochondrial preparations with clorgyline. Panel III shows labelled peptides exclusively from the A form, as binding of  $^3\text{H}$ -pargyline to the active site of type B MAO was blocked by preincubation with deprenyl. The  $^3\text{H}$ -pargyline-labelled peptides displayed in panel IV resulted from limited proteolysis of MAO from rat glioma line C6. This cell line has only type A MAO activity<sup>21</sup> and was derived from a different strain of rats from the  $\text{MH}_1\text{C}_1$  line.

A peptide fragment of approximately MW 29,000 is prominent in the maps shown in panels I and II, but is missing from the maps in panels III and IV. This fragment was present only when the B form of MAO was labelled. The other three fragments in the maps were common to both A and B forms of MAO. In preliminary studies the proteolytic enzyme papain was found to generate four  $^3\text{H}$ -pargyline-labelled peptide bands (MW  $> 10,000$ ) which were common to both forms of MAO. Pro-

teolysis by chymotrypsin produced two labelled bands common to A and B and one band unique to the A form (data not shown).

The flavin-containing polypeptides which underlie the A and B types of MAO activity in the rat are similar in MW<sup>14,15</sup>. Furthermore, radiolabelled MAO A and B from rat hepatoma and glioma cells have identical mobilities (M.R.C. Costa and X.O.B., unpublished) in a non-equilibrium pH gradient gel system<sup>22</sup>, showing they also have a similar charge. Discrete regions in these polypeptides were analysed using site-specific proteases in denaturing and solubilising conditions for MAO. The different peptide maps obtained for MAO A and B indicate differences in the location of the target sites for the protease, or in their susceptibility to proteolysis. These differences could result from divergent amino acid sequences or differential post-translational modifications. Such molecular differences may determine the association of these polypeptides with other molecules, and the final position and conformation of the enzyme within the mitochondrial membrane. An important hypothesis that is ruled out by these data is that the differences underlying the A and B types of MAO activity are solely due to



the membrane microenvironments of identical enzyme molecules.

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**Note added in proof:** Using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Callingham and Parkinson found that in rat tissues the pargyline binding polypeptides associated with type A and B MAO activities have MWs of 60,000 and 55,000, respectively (MAO Symposium, Midland, Michigan, 1979). We subsequently modified our SDS-PAGE technique and have now demonstrated a similar MW difference for these polypeptides in rat hepatoma cells, providing additional support for the conclusion that MAO A and B are distinct enzyme molecules.

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## Structure of two spliced mRNAs from the transforming region of human subgroup C adenoviruses

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The papova viruses and the human adenoviruses are widely used as a model system to study cell transformation *in vitro*. In subgroup C human adenoviruses, fragment *HpaI*-E, which comprises as little as 4.5% of the adenovirus type 5 (ad5) DNA, is sufficient for transformation of rat embryo cells<sup>1</sup>. Analysis of messenger RNAs (mRNAs) from the transforming region of adenoviruses type 2 (ad2) has identified several spliced mRNA species<sup>2-4</sup>. Promoter mapping studies indicate that the leftmost early region contains two separate transcription units, E1A and E1B (ref. 5) (Fig. 1a). Region E1A is approximately equivalent

*HpaI*-E. The complete nucleotide sequence of the *HpaI*-E fragment of ad5 was recently reported<sup>6</sup>. However, the spliced nature of early adenovirus mRNAs prevents a prediction of the amino acid sequence of the corresponding polypeptides directly from the DNA sequence. To study the structure of early ad2 mRNAs at the nucleotide level, we have used molecular cloning procedures to amplify the appropriate mRNA sequences. In this report, clones corresponding to the 12S and 13S mRNA from region E1A (Fig. 1c) have been isolated and characterised by hybridisation and sequence analysis. Our results enable us to predict the primary sequence of two related polypeptides from region E1A of human subgroup C adenoviruses.

To clone early ad2 mRNAs, double-stranded complementary DNA (cDNA) copies were inserted into the *Pst*I site of plasmid pBR322 after dG/dC tailing with terminal transferase. Clones containing ad2-specific sequences were identified by hybridisation<sup>7</sup>, using ad2 DNA or specific fragments of ad2 DNA as probes. In this way three clones were identified which contain sequences from region E1A of the ad2 genome. These clones are designated 147, 131 and 133. Plasmid DNA was extracted from these clones, labelled *in vitro* by nick translation and hybridised to *Sma*I fragments of ad2 DNA. Plasmid DNA from clones 131 and 133 hybridised to both fragments *Sma*I-J and *Sma*I-E (map coordinates 0-2.9 and 2.9-10.7) whereas clone 147 hybridised exclusively to fragment *Sma*I-E (Fig. 2).

The clones were further analysed by restriction enzyme cleavage using endonucleases *Hpa*I, *Xba*I, *Sma*I and *Pst*I. Because the cDNAs were inserted into the *Pst*I site of plasmid pBR322 by dG/dC tailing, endonuclease *Pst*I will excise the cDNA insert. Clone 147 contained a 440-nucleotide-long insert which lacked the cleavage site for *Sma*I whereas *Hpa*I cleavage resulted in two fragments, 130 and 310 base pairs long. From these results we conclude that clone 147 contains sequences corresponding to the 3'-end of a mRNA from region E1A (see Fig. 1a-c).

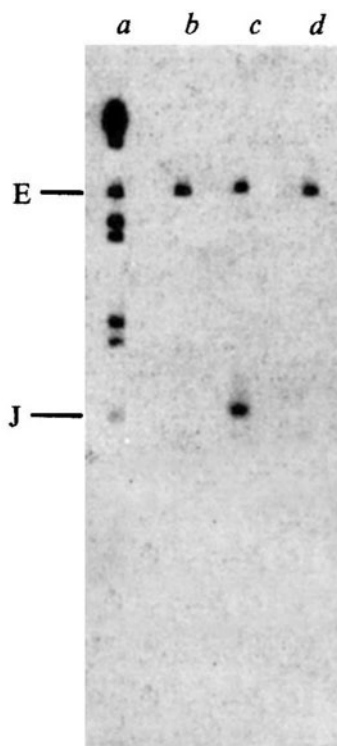
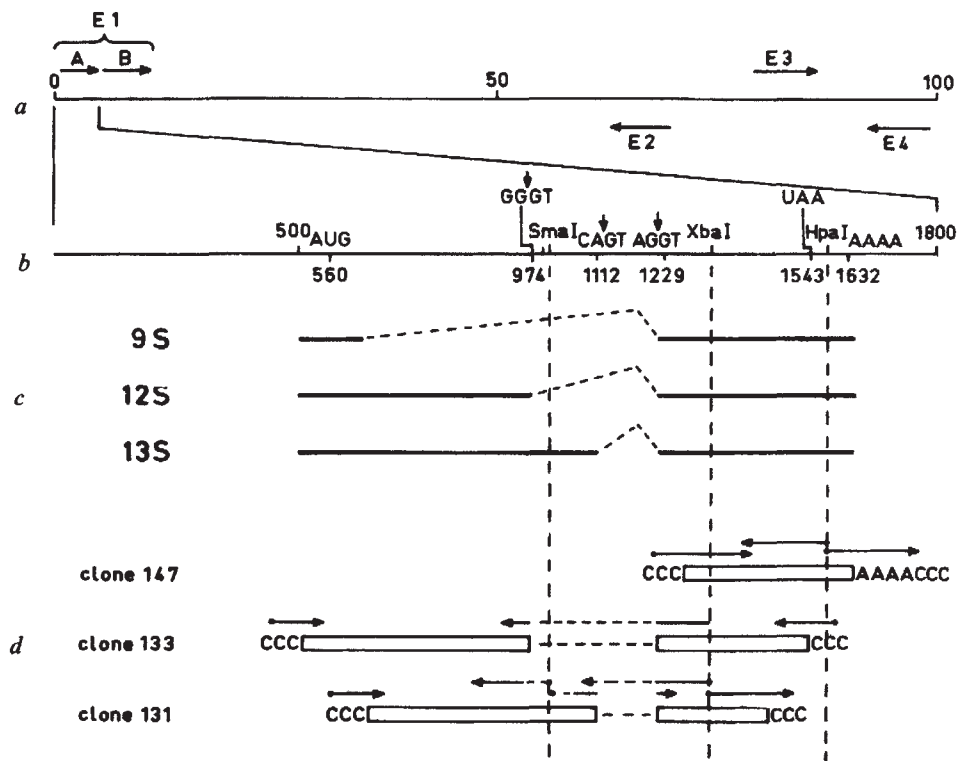
Clone 133 contained a cDNA insert 840 base pairs long that lacked a cleavage site for *Sma*I, whereas *Xba*I cleavage gave two fragments 620 and 220 base pairs long. These results together with the hybridisation results indicate that clone 133 corresponds to the 12S mRNA of region E1A (Fig. 1c).

The cDNA insert in clone 131 was approximately 650 base pairs long, and was cleaved precisely in the middle by *Sma*I. Cleavage with *Xba*I resulted in two fragments, 510 and 140 base pairs long. The presence of a cleavage site for *Sma*I in the cDNA insert suggests that clone 131 is related to the 13S mRNA of region E1A although it is not a complete copy of this mRNA.

To study the structure of the cloned mRNAs at the nucleotide level, sequence analysis according to the Maxam and Gilbert<sup>8</sup> procedure was carried out. The strategy for our sequencing approach is shown in Fig. 1b and d, which also outlines the results. The proposed sequence for the 12S and 13S mRNAs from region E1A is shown in Fig. 3. Although none of the clones contains a complete copy of the 12S or 13S mRNA, it was possible to combine our sequence results from the clones with the DNA sequence for the left-hand end of ad5 DNA<sup>6</sup> to deduce the structure of the two mRNAs. Both ad2 and ad5 belong to subgroup C adenoviruses and their transforming regions are nearly identical<sup>9</sup>. From our clones we have collected sequence information comprising more than 500 nucleotides (Fig. 1d) and have found only six base-pair changes between ad2 and ad5 DNA (Fig. 3). The sequence at positions where splicing takes place are in all cases identical between ad2 and ad5. Thus, it is possible to combine our sequence information from the clones with the genomic DNA sequence of ad5 (ref. 6) to deduce the structure of the 12S and 13S mRNAs from region E1A. The structure is based on the assumption that the 12S and 13S RNAs have common 5'- and 3'-ends.

The splicing events which must occur during maturation of the 12S and 13S mRNAs result in the deletion of a markedly AT-rich region, between nucleotides 1,113 and 1,228, which contains many stop codons. In this respect the splice has the same function as the splice in the mRNA for the large T-antigen

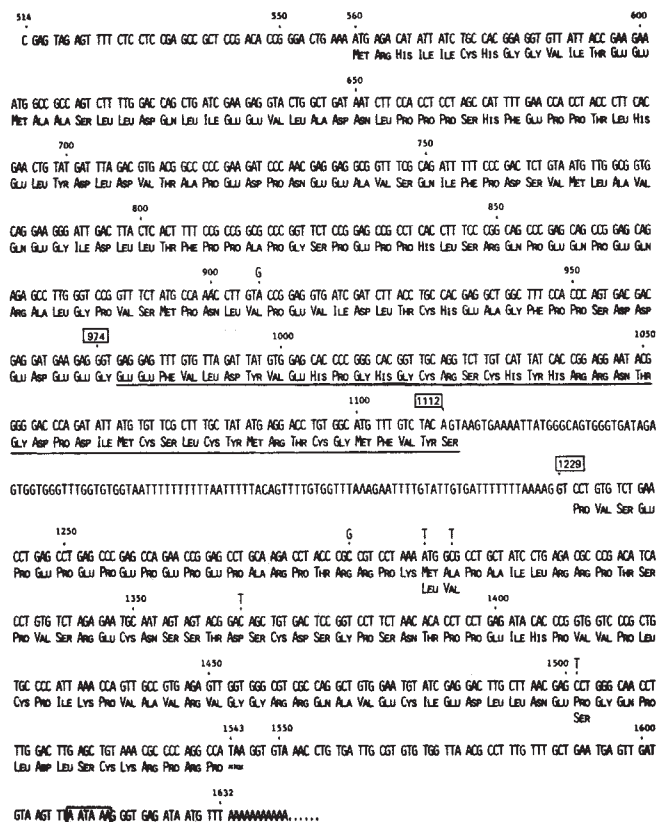
**Fig. 1** A schematic drawing of the ad2 genome, showing the four regions which are transcribed early after infection.<sup>18,19</sup> Region E1 contains two promoters which divide it into E1A and E1B (ref. 5). An enlargement of region E1A, indicating cleavage sites for selected restriction enzymes. The locations of the initiator AUG, the termination codon, the poly(A) addition site and the splice points for the 12S and 13S mRNAs are indicated. The nucleotide sequences around the splice points are also shown. Due to the presence of tandem repeats at the splice points (GT in the 13S mRNA and GGT in the 12S mRNA) it is not possible to pinpoint precisely the positions where splicing has taken place. However, by following the 'GT-AG' rule (ref. 20) tentative locations for the splice points can be established and are indicated by arrows. Following this assumption, nucleotide 974 is connected to nucleotide 1,229 in the 12S mRNA, and in the 13S mRNA nucleotide 1,112 is connected to nucleotide 1,229. *c*, structure of the 9S, 12S and 13S mRNAs as determined by electron microscopy<sup>4</sup> and *S*<sub>1</sub> nuclease mapping<sup>2</sup>. *d*, The boxes indicate sequences which are present in clones 147, 133 and 131. The clones were prepared in the following way. Complementary DNA (cDNA) from 10 µg of poly(A)-containing RNA, prepared as described previously<sup>21</sup> was synthesised with avian myeloblastosis virus (AMV) DNA polymerase, using oligo(dT)<sub>12-18</sub> as a primer in 0.2 ml of a reaction mixture containing 50 mM Tris-HCl, pH 8.2, 10 mM KCl, 75 mM dithiothreitol, 10 mM MgCl<sub>2</sub>, 4 mM sodium pyrophosphate, 0.05 mM [ $\alpha$ -<sup>32</sup>P]ATP and 0.02 mM dGTP, dCTP and dTTP. The RNA template was removed by hydrolysis in 0.3 M NaOH for 2 h at 50 °C. A second DNA strand was synthesised either with DNA polymerase I or AMV DNA polymerase in the same reaction mixture as described above, without pyrophosphate. The resulting hairpin loop was eliminated by *S*<sub>1</sub> treatment. The double-stranded cDNA was inserted into the *Pst*I site of the pBR322 plasmid after dG/dC tailing with terminal transferase. The recombinant plasmids were propagated in the DP50 strain of *Escherichia coli* in EK 2 host-vector conditions. The experiments were carried out in the P3 facilities available at the Pasteur Institute, Paris. Details of our cloning procedure will be described elsewhere<sup>15</sup>. Colonies containing ad2-specific sequences were identified by hybridisation<sup>7</sup>. The arrows indicate regions which were sequenced by the Maxam and Gilbert<sup>8</sup> procedure.



**Fig. 2** Hybridisation between <sup>32</sup>P-labelled DNA from clones 147 (*b*), 133 (*c*), 131 (*d*) and *Sma*I fragments of ad2 DNA. DNA from the recombinant plasmids was labelled *in vitro* by nick translation. Ad2 DNA was cleaved with *Sma*I and transferred to nitrocellulose<sup>22</sup>. Hybridisation was visualised by autoradiography. <sup>32</sup>P-labelled and ad2 DNA was hybridised to indicate the position of all fragments (*a*).

of SV40 (refs 10–12). Also, like the situation for SV40 T-antigen mRNA<sup>13</sup>, the 5'- and 3'-parts of both mRNAs will be translated from different reading frames. In this way the coding capacity of region E1A is used in the most efficient way. The polypeptides which are specified by the 13S and 12S mRNAs will be 288 and 242 amino acids long (Fig. 3), with molecular weights of 32,000 and 26,000, respectively, assuming that the AUG at position 560 is used for initiation. Both polypeptides will have identical amino- and carboxy-terminal ends, their only difference being a deletion of 46 internal amino acids from the polypeptide, specified by the shorter mRNA (Fig. 3). Both peptides are unusually rich in proline and glutamic acid (Fig. 3); this may explain why their molecular weights have been overestimated by SDS-polyacrylamide gel electrophoresis<sup>14</sup>. By *in vitro* translation of mRNAs from the extreme left-hand end of the ad2 genome, four polypeptides in the molecular weight range of 42–53,000 have been detected<sup>14</sup>. Thus, more polypeptides have been assigned to region E1A than can be accounted for by the mRNAs which have been observed by electron microscopy<sup>4</sup> and the *S*<sub>1</sub> nuclease assay<sup>2</sup>. This could be due to post-translational modification or processing of the primary translation products. However, we cannot exclude the possibility that additional mRNAs are specified by region E1A which have such small differences in the positions of their splices that they have escaped detection. Sequence analysis of additional clones is clearly required to resolve this problem. The 3'-end of region 1A mRNAs has been positioned to nucleotide 1,632. However, as two As follow the T in the DNA sequence, it is not possible to predict precisely where the poly(A) addition occurred. We have made a similar observation for the poly(A) addition site in the mRNA for adenovirus polypeptide IX<sup>15</sup> and the presence of two or more As at the poly(A) addition site may be a common feature. Our results do not exclude the presence of





**Fig. 3** The proposed structure for the 13S mRNA as deduced from our analysis of the mRNA clones together with the sequence for the left hand end of ad5 DNA<sup>6</sup> (J. Maat *et al.*, personal communication). The sequence corresponds to the 13S mRNA from ad5. Differences between the ad2 and ad5 sequences as noted in our present study are indicated. The 5'-end of region E1A mRNAs is not defined by our cloned sequences. Nucleotide 514 is the first nucleotide present in clone 133. The true 5'-end is probably located a few nucleotides upstream from this position. The sequence which is absent in the 12S mRNA is underlined. The hexanucleotide, present close to the poly(A) junction of eukaryotic mRNAs<sup>16</sup>, is indicated by the box. Nucleotides which become connected after splicing are shown in boxes (see also Fig. 1b). The nucleotide sequence is indexed according to the method of van Ormondt *et al.*<sup>6</sup>.

other poly(A) addition sites for region 1A mRNAs, as only one poly(A) junction has been sequenced so far. This seems, however, unlikely, because a single hexanucleotide sequence, AAUAAA, is present in the DNA sequence<sup>6</sup> and this sequence has been found to precede the poly(A) junctions of all eukaryotic mRNAs<sup>16</sup>. Sequence analysis of clone 133 places the 5'-end of region 1A mRNAs at nucleotide 514 in the sequence of van Ormondt *et al.*<sup>6</sup>. It is, however, conceivable that a few nucleotides are missing from this clone, as the second strand of the cDNA was synthesised by looping back the first strand. Furthermore, a promoter-like sequence, TATTTATA, starts 39 nucleotides upstream from position 514. Such sequences are usually found 23–26 nucleotides preceding the cap-site (D. Hogness, personal communication and ref. 17).

Independent of this investigation, Baker and Ziff (manuscript in preparation) have identified and analysed the capped T<sub>1</sub> oligonucleotide from region E1A mRNAs. Their results indicate that nucleotide 499 in the sequence of van Ormondt *et al.*<sup>6</sup> corresponds to the 5'-end of mRNAs from region E1A. Thus, the first 15 nucleotides of the 12S mRNA are missing from clone 133.

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## Inter- and intraspecific variation in restriction maps of *Drosophila* mitochondrial DNAs

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Mitochondrial (mt) DNAs have been observed to be polymorphic for cleavage sites of various restriction endonucleases<sup>1–4</sup>. Although these studies involved a variety of organisms, they were insufficient for the estimation of the level of genetic variation in natural populations. mtDNAs of various *Drosophila* species are heterogeneous in length, with molecular weights (MWs) ranging from 9.90 to 12.35 × 10<sup>6</sup>. The mtDNA molecule of *Drosophila melanogaster* contains a long A + T-rich region representing 25% of the circle length<sup>5,6</sup>. A comparison of mtDNAs from different species of *Drosophila* by electron microscope partial denaturation mapping has shown that the size of the A + T-rich region varies among different species<sup>5</sup>. We have previously presented electron microscope heteroduplex analysis of the mtDNAs of *D. melanogaster*, *Drosophila simulans* and *Drosophila virilis* and showed that the sequence divergence was apparently greatest in the A + T-rich region, whereas the remainder of the molecule was much more evolutionarily conserved. The cleavage map of the *D. melanogaster* mtDNA molecule has been established using various restriction endonucleases<sup>6–8</sup>. The ribosomal RNA genes (rRNAs) and the A + T-rich region have been positioned on this map. We now present the restriction endonuclease maps of the mtDNAs of three species of *Drosophila*. We also report the results of a systematic survey of restriction site variation in the mtDNAs of natural populations of these species and make a preliminary comparison with the level of variation of nuclear genes. Our data suggest that the mitochondrial genome may evolve in a similar way to allozymic variation.

In this study, we have mapped the restriction sites in the mtDNA molecules of *D. virilis*, *D. melanogaster* and *D. simulans* using the enzymes *EcoRI*, *HindIII* and *HpaII*. These enzymes make a small number of cuts in these mtDNAs and are therefore valuable in mapping the cleavage sites and in definitive identification of the conserved sites. mtDNAs were purified by

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**Table 1** Sizes of the restriction fragments of *Drosophila* mtDNAs

| Fragment | <i>Hind</i> III |             |             | <i>Hae</i> III |             |             | <i>Eco</i> RI |             |             | <i>Hpa</i> II |             |             |
|----------|-----------------|-------------|-------------|----------------|-------------|-------------|---------------|-------------|-------------|---------------|-------------|-------------|
|          | <i>D.m.</i>     | <i>D.s.</i> | <i>D.v.</i> | <i>D.m.</i>    | <i>D.s.</i> | <i>D.v.</i> | <i>D.m.</i>   | <i>D.s.</i> | <i>D.v.</i> | <i>D.m.</i>   | <i>D.s.</i> | <i>D.v.</i> |
| A        | 7.39            | 7.39        | 6.14        | 8.88           | 6.14        | 14.85       | 11.33         | 7.80        | 9.47        | 9.70          | 14.24       | 10.15       |
| B        | 4.90            | 4.90        | 5.61        | 5.52           | 5.42        |             | 5.24          | 4.35        | 5.18        | 3.46          | 2.06        | 5.30        |
| C        | 4.67            | 4.67        | 2.09        | 3.35           | 5.42        |             | 1.58          | 1.67        | 0.83        | 3.00          | 1.76        |             |
| D        | 0.43*           | 0.43*       | 1.21        |                |             |             | 0.98          | 1.36        |             | 2.01          |             |             |
| E        |                 |             |             |                |             |             |               | 1.14        |             |               |             |             |
| F        |                 |             |             |                |             |             |               | 0.91        |             |               |             |             |
| G        |                 |             |             |                |             |             |               | 0.65        |             |               |             |             |
| Total    | 17.39           | 17.39       | 15.05       | 17.93          | 16.98       | 14.85       | 19.13         | 17.88       | 15.48       | 18.17         | 18.06       | 15.45       |

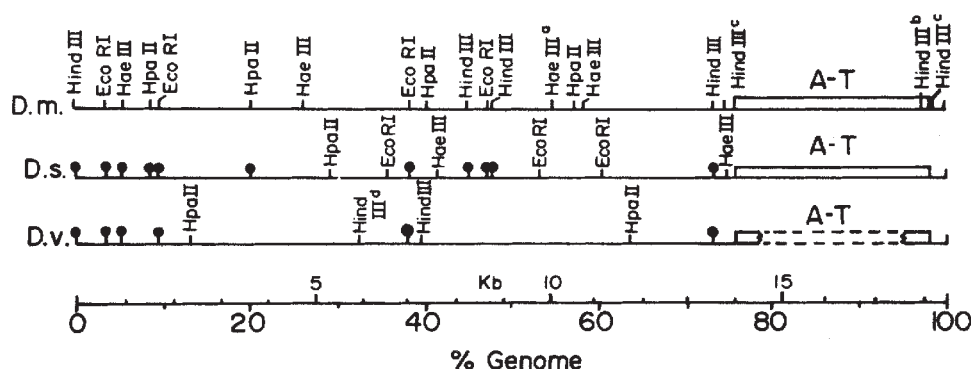
Fragment sizes are given in kilobases. The sizes of the fragments were determined from the agarose gels except as mentioned above. The sizes of *D. melanogaster* and *D. simulans* mtDNAs as determined by electron microscopy are 18.61 and 18.53 kilobases, respectively; that of *D. virilis* mtDNA is 15.86 kilobases. *D.m.*, *D.s.* and *D.v.* refer to *D. melanogaster*, *D. simulans* and *D. virilis* mtDNAs, respectively.

\* Measured from polyacrylamide gels.

two cycles of CsCl-ethidium bromide centrifugation and digested with appropriate restriction endonucleases as described previously<sup>7</sup>. The sizes of the resulting fragments were determined from the calibrated agarose or polyacrylamide gels and by electron microscopy using ΦX174 replication form (RF) II DNA as an internal standard. Table 1 lists the sizes of the *Eco*RI, *Hind*III, *Hae*III and *Hpa*II restriction fragments of *Drosophila* mtDNAs. We have determined the relative positions of these restriction fragments on the mtDNA molecules by analysing the products of various double-enzyme digests. Thus, a total of 10 restriction sites were mapped on the *D. virilis* mtDNA molecule, whereas a total of 15 and 17 restriction sites were mapped on the mtDNA molecules of *D. melanogaster* and *D. simulans*, respectively. Figure 1 shows the cleavage maps of these mtDNAs. The cleavage maps of the *D. melanogaster* mtDNA molecule showing the positions of *Eco*RI, *Hind*III and *Hae*III sites have been reported previously<sup>6,8</sup>. Our map of *D. melanogaster* mtDNA molecule agrees with the published maps. The distribution of restriction sites is similar in two closely related species, *D. melanogaster* and *D. simulans*, but is markedly different in *D. virilis*, which is more distantly related to the other two species. *D. melanogaster* and *D. simulans* mtDNA molecules are similar in size<sup>7</sup> and share 11 restriction sites. The *D. virilis* mtDNA molecule is 2.75 kilobases shorter and shares only six restriction sites (see Fig. 1). One striking feature of *Drosophila* mtDNA maps is that four restriction sites proximal to the A+T-rich region are conserved in mtDNAs of all three species. Klukas and Dawid<sup>6</sup> have mapped mt-rRNA genes in this region of *D. melanogaster* mtDNA. It is therefore evident that the restriction sites within the rRNA genes of *Drosophila* mtDNAs are conserved. We have also mapped the A+T-rich region of each mtDNA molecule on the cleavage map by partial denaturation mapping and heteroduplex analysis of the *Hind*III

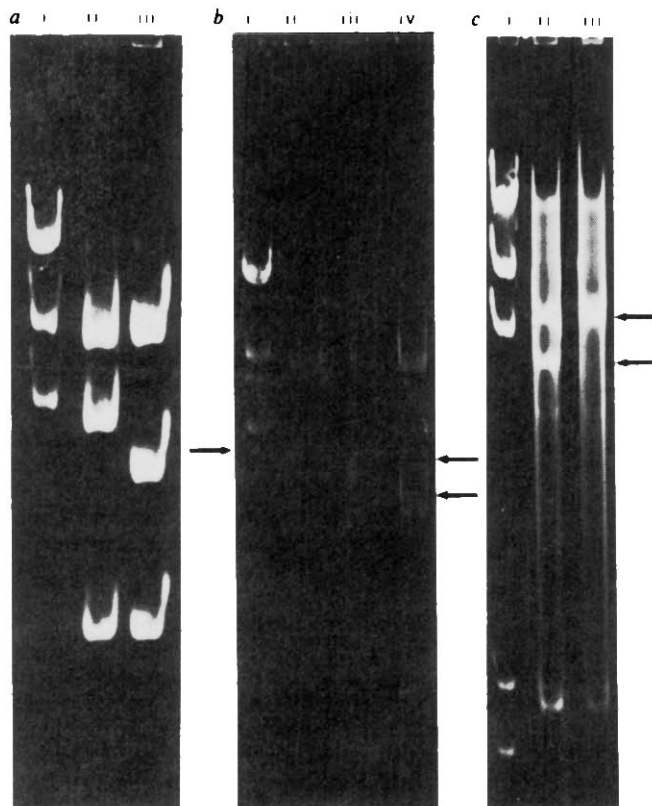
and *Hae*III fragments of mtDNAs (see Fig. 1). It is evident that the relative positions of the A+T-rich regions are similar in mtDNA molecules of all three species. From the maps presented, it is clear that the A+T-rich region does not contain any restriction sites. *Alu*I, which recognises the sequence 5' AGCT 3', cuts *D. melanogaster* and *D. simulans* mtDNA molecules into approximately 18 fragments, but fails to cut the A+T-rich region. This region therefore contains little or no G and C. Using the maximum likelihood estimator of Kaplan *et al.*<sup>9</sup>, we have estimated a 6% sequence divergence between *D. melanogaster* and *D. simulans* mtDNAs outside the A+T-rich region and the rRNA genes. However, the restriction map of *D. virilis* mtDNA is completely different from *D. melanogaster* and *D. simulans* mtDNAs outside the rRNA genes so that no further estimations could be made. However, some of the restriction sites surveyed might be appropriately modified in these native mtDNAs, leading to spurious conclusions concerning the presence of the sequence. In the absence of any direct evidence with respect to this question the analysis mentioned above and subsequent analysis of population data (below) cannot take it into account.

Ten isofemale lines of *D. melanogaster* were established simultaneously from a wild collection made in Raleigh, North Carolina, in 1977. Ten isofemale lines of *D. virilis* were also established from two collections made in Sapporo and Moji, Japan, in 1977 (five lines each). Five isofemale lines of *D. simulans* came from a collection made in Mishima, Japan, in 1977. The flies were cultured, the mtDNAs purified from the second and third instar larvae, and restriction sites mapped. Within each isofemale line mtDNA molecules always appeared homogeneous with respect to their restriction maps. Three variable sites were found among *D. melanogaster* lines (see Fig. 1). Figure 2a and b show the electrophoretic patterns of the restriction enzyme digest of different mtDNAs. These were



**Fig. 1** The *Hind*III, *Hae*III, *Eco*RI and *Hpa*II restriction maps of *D. melanogaster* (*D.m.*), *D. simulans* (*D.s.*) and *D. virilis* (*D.v.*) mtDNAs. Units are % of genome and kilobases. Sites with superscripts are variable, as indicated in Fig. 3. The alternative positions of *Hind*III<sup>a</sup> are indicated. *D. melanogaster* and *D. simulans* mtDNAs are 18.6 kilobase pairs in length. *D. virilis* mtDNA has 15.8 kilobase pairs. The reduction in the size of the A+T-rich region in *D. virilis* mtDNA has been indicated by broken lines (====). ● Represents the restriction sites in *D. simulans* and *D. virilis* mtDNAs which are conserved in *D. melanogaster* mtDNA. Circular maps have been linearised to show the conserved restriction sites.





**Fig. 2** Restriction analysis of *Drosophila* mtDNAs. *a*, 1.2% agarose gel of *Hind*III-digested  $\lambda$  DNA (i), *Hae*III-digested *D. melanogaster* mtDNA without *Hae*III<sup>a</sup> site (ii) and with the *Hae*III<sup>a</sup> site (iii). *b*, 1.2% agarose gel of *Hind*III-digested  $\lambda$  DNA (i), *Hind*III-digested *D. melanogaster* mtDNA without sites *Hind*III<sup>b</sup> or *Hind*III<sup>c</sup> (ii), with *Hind*III<sup>b</sup> and without *Hind*III<sup>c</sup> (iii) and with *Hind*III<sup>c</sup> (iv). The lack of fluorescence of the middle band is probably due to long poly(dA)·poly(dT) segments. *c*, A 1.2% agarose gel of *Hind*III-digested  $\lambda$  DNA (i), *Hind*III-digested *D. virilis* mtDNA with the *Hind*III<sup>a</sup> restriction site (ii) and without the *Hind*III<sup>a</sup> site (iii). The gels were stained with ethidium bromide and photographed on Polaroid films using short-wave UV source.

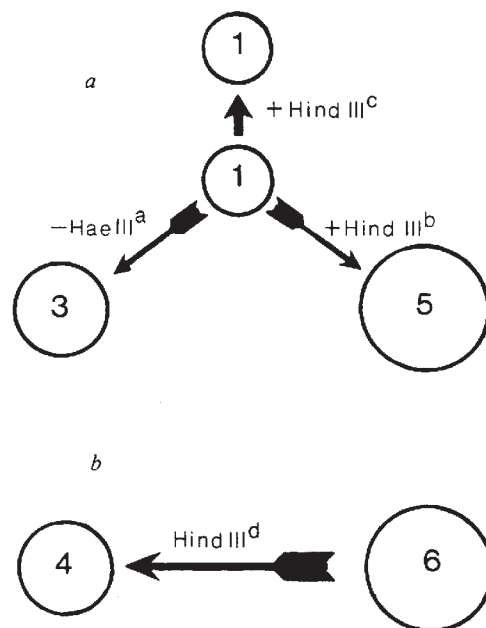
distributed among the various lines as shown in Fig. 3a. The other variable site occurred among *D. virilis* lines as indicated in Figs 1 and 3b. Figure 2c shows the two *Hind*III patterns observed in *D. virilis*. The two sampling locations gave equal numbers of the two types of *D. virilis* mtDNA. No variation was observed among the lines of *D. simulans*.

Other than these data, the only information available on the intraspecific variability of *Drosophila* mtDNA restriction maps is from the 'standard' lines used in earlier studies. The standard line of *D. melanogaster* (D.m.1) used in our earlier work was collected in Raleigh, but 2 yr earlier than those reported above. The restriction map of this line for the four restriction enzymes is identical to that of the second most frequent class (see Fig. 3a). Restriction maps obtained by other laboratories on *D. melanogaster* mtDNA with *Hind*III, *Hae*III and *Eco*RI also fall into this class<sup>6,8</sup>. This mtDNA restriction map seems to be widespread and temporarily stable. The *D. virilis* line described in our previous studies<sup>7</sup> was also from Japan but collected 1 yr earlier in Fukuoka. We examined two lines from that collection, one of which had our standard and the other the variant pattern (Fig. 2c), suggesting temporal and geographical stability for this polymorphism. Finally, our *D. simulans* standard was derived from Raleigh, whereas our population sample (above) was collected several years later in Japan. The uniformity of the restriction maps in *D. simulans* is in striking contrast to the variability observed in *D. melanogaster*.

We can attempt to answer two questions with these results. The first concerns the significance of the interspecific differences in restriction map variability and the second compares the amount of variation in mtDNA and nuclear DNA. Unfortunately, there has been no systematic survey on the population level of restriction map variation in nuclear DNA. To investigate these matters, we must first determine a metric of restriction map variability that lends itself to analysis (the product of the population size and mutation rate).

Fundamentally, the population genetics of restriction maps is a special case of the 'finite sites model' (ref. 9). Also, the finite sites model is well approximated by the 'infinite alleles model' when the amount of variation relative to that possible is small<sup>10</sup>. Assuming the non-recurrent neutral mutation, finite population model<sup>11</sup> (infinite alleles model), we can estimate the pertinent parameter by two methods. If  $N_e$  is the effective population size (number of gene copies), and  $\mu$  the mutation rate<sup>11</sup>, either we can set  $1/(2N_e\mu + 1)$  equal to the 'expected homozygosity', that is, the sum of squared frequencies<sup>11</sup>, or  $2N_e\mu$  can be estimated by the maximum likelihood method of Ewens<sup>12</sup>. Estimates from these methods do not differ greatly (*D. melanogaster*, 3.6 or 2.0; *D. virilis*, 1.8 or 0.8; *D. simulans*, 0.0). When these values are tested using the sampling statistics of Ewens<sup>12</sup>, the three species do not differ significantly.

To compare the mtDNA and allozyme data, each must be converted into a common unit of genetic variation. In our estimates of  $2N_e\mu$ , the mutation rate,  $\mu$ , is the rate per generation of nucleotide changes that create new restriction sites or destroy pre-existing ones, that is, the rate of nucleotide substitutions that create new restriction maps. As pointed out above, if the extant variation is small (as it is in these cases) virtually all site changes will be new. If we assume a random nucleotide sequence and random mutation, the proportion of nucleotide substitutions that change restriction sites can be estimated<sup>7</sup>. In our case, it is 0.02 for the region of 12,000 base pairs excluding the A+T-rich DNA and that coding for the mt-rRNA. We exclude these regions because the A+T-rich region contains no restriction sites (GC content too low) and the rRNAs are much more conservative evolutionarily. On a per nucleotide basis,  $2N_e\mu$  is then between 0.008 [ $2.0/(0.02 \times 12,000)$ ] and 0.0, with a mean of 0.003.



**Fig. 3** *a* Represents the numbers of different mtDNAs found among the *D. melanogaster* isofemale lines and the substitutional paths between them. *b* Is a similar presentation of the mtDNAs of the *D. virilis* isofemale lines.

As there are no comparable estimates of restriction map variability in nuclear DNA, we must attempt to use electrophoretic data on population variability. Starting with an estimate of 0.04–0.14 for expected heterozygosity<sup>13,14</sup>, we estimate  $2N_e\mu$  to be 0.04–0.16 (ref. 11). If we assume that one-quarter of all nucleotide substitutions change the amino acid<sup>15</sup> and one-third of amino acid substitutions change the electrophoretic mobility, then  $\mu$  in the above estimate is 0.08 of the total nucleotide mutation rate. Assuming 1,000 nucleotide positions per protein, this yields an estimate for  $2N_e\mu$  of 0.0005–0.002 on a per nucleotide basis. Note that the electrophoretic techniques of detection of genic variation in proteins are more sensitive than the use of these four restriction enzymes (8% compared with 2% of the nucleotide substitutions). This is partly due to the low GC content of *Drosophila* mtDNAs.

Thus, we estimate  $2N_e\mu$  for mtDNA at 0.003 (0–0.008) and for allozyme loci at 0.0005–0.002. Even considering the fact that  $N_e$  should be twice as large for nuclear (diploid) genes, there do not seem to be any significant differences in genetic variation between allozymes and mtDNAs. Whether this picture will survive more detailed scrutiny is unknown, but it seems likely that the quality of data on molecular genetic variation in natural populations will change significantly.

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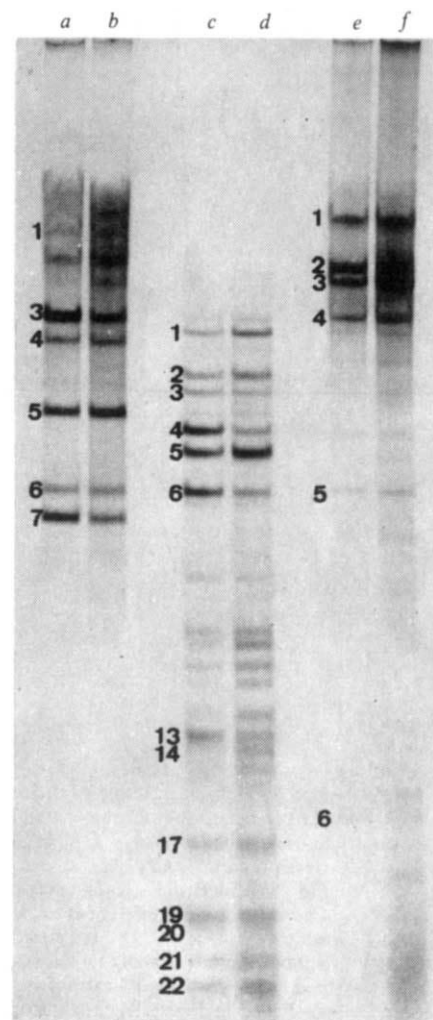
## Structure of the region of the replication origin of the *Bacillus subtilis* chromosome

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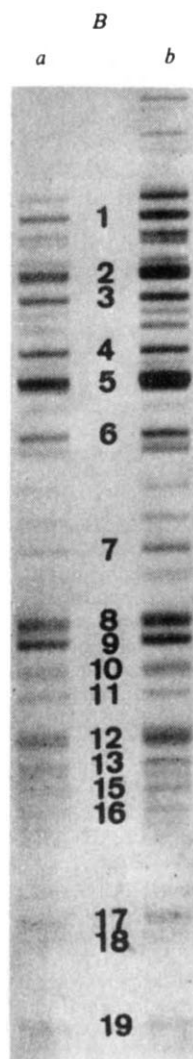
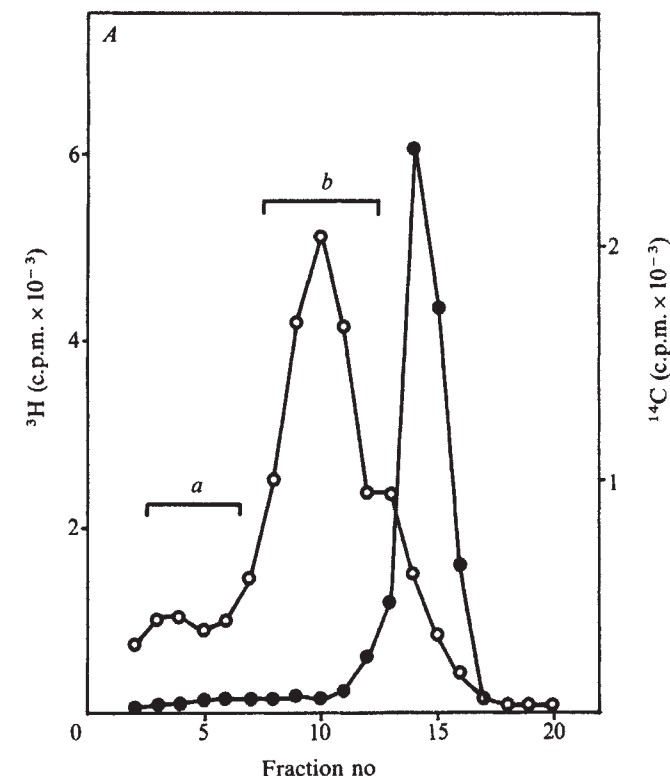
The chromosome of a Gram-positive bacterium, *Bacillus subtilis*, binds tightly to the cell membrane<sup>1–3</sup> probably through a DNA–protein–RNA complex formed specifically near the origin of replication<sup>4,5</sup>. Characterisation of this complex may reveal the role of the cell surface in regulating the initiation of DNA replication in bacteria. As a first step towards this goal, we have constructed a map of the restriction enzyme cleavage sites at the region of the origin of replication of the *B. subtilis* chromosome. Adopting the methodology originally used by Marsh and Worcel in *Escherichia coli*<sup>6</sup>, the region of the origin of replication of the chromosome was labelled specifically with a radioisotope which was then used to characterise DNA fragments produced by various combinations of restriction enzyme. In the study reported here, cleavage sites by three enzymes were identified within the chromosomal fragment of molecular weight  $4 \times 10^7$  at the origin region.

A thymine-requiring mutant of *B. subtilis*, CRK2000 (*trp*, *leu*, *thy*)<sup>7</sup>, and its *ts-dna* mutant derivative, CRK2001 (*trp*, *leu*, *thy*, *din*<sup>ts</sup>)<sup>8</sup> which is defective in the initiation of DNA replication at 47 °C, were used to label the DNA at the origin of replication. Initiation of the replication cycle was synchronised either by germinating spores in the absence of thymine for at least 2 h or by incubating the *ts-dna* mutant cells at 47 °C for 1 h followed by incubation at 30 °C in the absence of thymine. In both cases addition of <sup>3</sup>H-thymidine (dThd) of high specific activity was assured to initiate chromosomal replication from the origin of replication and to label the origin region of the chromosome selectively<sup>4</sup>. The labelled DNA was purified and digested with



**Fig. 1** Restriction endonuclease analysis of DNA, pulse labelled during the initiation of replication. Spores of *B. subtilis* CRK2000 (*leu*, *trp*, *thy*)<sup>7</sup> were germinated at 37 °C in a synthetic medium, GM11 (ref. 1), in the absence of thymine for 150 min, and then labelled with <sup>3</sup>H-dThd (Radiochemical Centre, Amersham), 10  $\mu$ Ci per 50 ng per ml, for 1 min (b, d, f). Novobiocin (Boehringer, Mannheim, 100  $\mu$ g ml<sup>-1</sup>) was added to a separate sample 5 min before the spores were labelled with <sup>3</sup>H-dThd for 8 min (a, c, e). Novobiocin reduced the rate of DNA replication to 7.5% of the control rate. The labelling was stopped by pouring the culture into an equal volume of ethanol–phenol (75% ethanol, 2% phenol in 21 mM Na-acetate buffer, pH 5.1, and 2 mM EDTA). Cells were collected by centrifugation, lysed with 1 mg ml<sup>-1</sup> lysozyme in a lysis buffer (20 mM Tris-HCl, pH 8.1, 100 mM KCl, 1 mM EDTA) for 30 min and then treated with 1% Sarkosyl (Geigy) and 500  $\mu$ g ml<sup>-1</sup> pronase E (Kaken Kagaku). DNA was extracted and purified by repeating the phenol extraction and precipitation by ethanol in the lysis buffer. Purified DNA was digested with *Bam*HI (prepared as described previously<sup>11</sup>) (a, b), *Eco*RI (Miles) (c, d) or with *Sma*I (e, f) according to standard methods. Fragments were separated by electrophoresis in 0.7% agarose gel (Seakem) at 125 V for 16 h and detected by fluorography as reported by Marsh and Worcel<sup>6</sup>.





**Fig. 2** Density labelling of the origin region of the chromosome. **A**, Spores, labelled with  $^{14}\text{C}$ -thymine, were prepared<sup>4</sup> and germinated as in Fig. 1.  $^3\text{H}$ -BrdU (Radiochemical Centre), 5  $\mu\text{Ci}$  per 0.5  $\mu\text{g}$  per ml, was added to start DNA replication. After 15 min the labelling was stopped and DNA was extracted as described in Fig. 1 legend. Purified DNA was mixed with CsCl in a buffer (25 mM Tris-HCl, pH 7.5, and 1 mM EDTA) at  $\rho = 1.70$  and centrifuged at 35,000 r.p.m. for 40 h at 20  $^{\circ}\text{C}$ . Samples were fractionated from the bottom of the tube and the radioactivity in the DNA fragments was measured as reported previously<sup>4</sup>.  $\circ$ ,  $^3\text{H}$ -BrdU;  $\bullet$ ,  $^{14}\text{C}$ -thymine. **B**, Fractions in *a* and *b* regions in **A** were collected and precipitated in ethanol with *E. coli* tRNA as a carrier. The precipitates were digested with *Eco*RI and subjected to electrophoresis as in Fig. 1. Fragments are numbered as in Fig. 1c, d.

various restriction endonucleases, and the resultant  $^3\text{H}$ -labelled fragments were analysed by agarose gel electrophoresis and fluorography.

Several discrete bands of fragments were detected in all enzyme digests. Figure 1 shows fragments produced by *Sma*I, *Eco*RI and *Bam*HI of the pulse-labelled DNA during synchronous initiation by germinating spores. The number of detectable bands increased with the length of the labelling period (details of the time course are reported in the accompanying paper<sup>9</sup>). Essentially identical fragment patterns were obtained when DNA was labelled during the synchronous initiation by *ts-dna* mutant (data not shown). To confirm that the label was incorporated by semi-conservative replication and not by a repair-type synthesis,  $^3\text{H}$ -bromodeoxyuridine (BrdU) was added in place of  $^3\text{H}$ -dThd to the germinating spores. A completely replicated DNA was collected by CsCl equilibrium density centrifugation and its restriction enzyme fragments were analysed. Figure 2 shows that (1) a short pulse with  $^3\text{H}$ -BrdU resulted in a density shift, indicating semi-conservative replication, and (2)  $^3\text{H}$ -BrdU-labelled fragments of the *Eco*RI digest were identical to those of  $^3\text{H}$ -dThd-labelled DNA.

Major bands of Fig. 1 were used for further analysis to construct the cleavage map. Each band produced by one enzyme was extracted, purified and then digested with the other two enzymes to make a thorough comparison between the three groups of fragments. Results of the analysis are summarised in Table 1 and a map of cleavage sites was constructed and is shown in Fig. 3. First, the relative location of fragments S6 and S2 were determined because fragment B3 overlapped these two *Sma*I fragments. As most of fragment B5 was derived from fragment S2, fragments B3 and B5 must be contiguous to each other. The third and largest *Sma*I fragment, S1, contained one *Bam*HI site, thus producing two *Bam*HI fragments, B1 and the major portion of B7. When fragment B7 was digested by *Sma*I three fragments were produced. The largest of these, of MW  $2.85 \times 10^6$ , corresponded to one of the two *Bam*HI fragments derived from S1, whereas the smallest fragment, of MW  $0.2 \times 10^6$ , corresponded to the smaller one of the two fragments derived from fragment S6 by *Bam*HI digestion. These results indicated that fragments S1 and S6 were contiguous to each other, with a fragment of  $0.5 \times 10^6$  MW inserted between them. Therefore, the relative locations of the *Sma*I fragments are S1– $0.5 \times 10^6$  MW fragment–S6–S2. From this result the order of the *Bam*HI fragments was deduced as B1–B7–B3–B5.

Once the cleavage sites of *Sma*I and *Bam*HI were mapped, *Eco*RI sites could be assigned as follows. Fragments E4 and E22 could be assigned as they were derived from one of the four *Bam*HI fragments and contained *Sma*I sites. In the same way fragments E6, E17 and E27 were located because they were derived from one of the three *Sma*I fragments and contained one *Bam*HI site. The space between fragments E6 and E22 matched precisely with E19, which is derived from fragment B7. Fragment E13 was located between E4 and E17 in the same manner. Fragments E5 and E20 can be mapped at any place within fragment B1. However, we set these fragments as contiguous to E6 because they were replicated much earlier than fragment E1. Relative positions of fragments E14, E21 and E26, all derived from B5, have not been determined. One of the major early replicating *Sma*I fragments, S3, whose *Bam*HI and *Eco*RI sites have been mapped as in Fig. 3, is most probably located close to S2. Assuming B5 and B6 to be contiguous to each other, there will be an additional *Sma*I fragment of MW  $\sim 1.8 \times 10^6$ , containing one *Bam*HI site, situated between fragments S2 and S3. A fragment of the corresponding MW can be seen above fragment S6 in Fig. 1. Therefore, it is most likely that fragments B5 and B6 are contiguous and so the map in Fig. 3 can be drawn continuously. However we cannot be certain of this until the additional, small *Sma*I fragment is fully characterised. The origin of replication is located within fragment B7, as described in the accompanying paper.

We have reported recently that a DNA segment near the origin of replication formed a specific complex (S-complex) with

Table 1 Results of enzyme analysis

| First cleavage | Fragment no. | MW ( $\times 10^6$ ) | Second cleavage       |                            |                                                                        |
|----------------|--------------|----------------------|-----------------------|----------------------------|------------------------------------------------------------------------|
|                |              |                      | <i>Bam</i> HI         | <i>Sma</i> I               | <i>Eco</i> RI                                                          |
| <i>Bam</i> HI  | 1            | 15                   |                       | ND                         | ND                                                                     |
|                | 3            | 8.2                  |                       | 6.7(S2), 1.3(S6)           | 4.7(E4), 1.9(E13), 0.86(E17), 0.40(E27)                                |
|                | 4            | 7.2                  |                       | Id                         | 4.9(E2), 2.1(E3)                                                       |
|                | 5            | 5.1                  |                       | 4.5(S2), 0.5               | 1.80(E14), 0.95(E21), 0.85, 0.55(E26), 0.45(E17) 0.30                  |
|                | 6            | 3.8                  |                       | 2.5(S3), 1.3               | 3.3(E3), 0.4                                                           |
|                | 7            | 3.4                  |                       | 2.85(S1), 0.50, 0.2(S6)    | 1.35(E6), 1.05(E19), 0.85(E22), 0.15(E27)                              |
|                |              |                      |                       |                            |                                                                        |
| <i>Sma</i> I   | 1            | 18                   | 15(B1), 2.9(B7)       |                            | 7.9(E1), 4.3(E5), 3.7(E6), 1.10(E19), 1.05(E20) 0.30(E22)*             |
|                | 2            | 11.6                 | 7.0(B3), 4.6(B5)      |                            | 3.8(E4), 1.9(E13), 1.8(E14), 1.4(E17), 0.95(E21) 0.56(E26), 0.37, 0.3* |
|                | 3            | 10.5                 | 7.2(B4), 2.7(B6), 0.4 |                            | 5.8(E2), 4.6(E3)                                                       |
|                | 6            | 1.5                  | 1.3(B3), 0.2(B7)      |                            | 0.98(E4), 0.54(E27), 0.06(E22)*                                        |
| <i>Eco</i> RI  | 1            | 8.0                  | ND                    | ND                         |                                                                        |
|                | 2            | 6.20                 | 5.0(B4), 0.7          | ND                         |                                                                        |
|                | 3            | 5.70                 | 3.4(B6), 2.0(B4)      | ND                         |                                                                        |
|                | 4            | 4.70                 | Id(B3)                | 3.70(S2), 1.0(S6)          |                                                                        |
|                | 5            | 4.30                 | Id(B1)                | Id(S1)                     |                                                                        |
|                | 6            | 3.70                 | 2.2(B1), 1.3(B7)      | Id(S1)                     |                                                                        |
|                | 13           | 1.90                 | Id(B3)                | Id(S2)                     |                                                                        |
|                | 14           | 1.80                 | ND                    | ND                         |                                                                        |
|                | 17           | 1.35                 | 0.90(B3), 0.45(B5)    | Id(S2)                     |                                                                        |
|                | 19           | 1.10                 | Id(B7)                | Id(S1)                     |                                                                        |
|                | 20           | 1.05                 | Id(B1)                | Id(S1)                     |                                                                        |
|                | 21           | 0.95                 | ND                    | ND                         |                                                                        |
|                | 22           | 0.86                 | Id(B7)                | 0.50, 0.30(S1)*, 0.06(S6)* |                                                                        |
|                | 26           | 0.56                 | ND                    | ND                         |                                                                        |
|                | 27           | 0.54                 | 0.40(B3), 0.14(B7)    | Id(S6)                     |                                                                        |

Fragment numbers are the same as those in Fig. 1. Each fragment was cut out from the gel and DNA was extracted with 4 M KI and purified by passing through a hydroxyapatite column as reported previously<sup>10</sup>. Each DNA sample was then digested with the other two enzymes and analysed by the same electrophoresis method as described in Fig. 1 legend. The MW of each fragment was determined by making parallel electrophoretic runs for <sup>3</sup>H-labelled standard fragments of known MW (*Eco*RI and *Hind*III digests of  $\lambda$  DNA). Id (indigestible) indicates that the fragment did not contain sites cleaved by the second enzyme concerned. ND, Not determined. By comparing each pair of doubly digested fragments of the same enzyme combinations but in reverse order, we determined the second source of fragment to which a given fragment belongs; these are indicated in parentheses.

\*Fragments assumed to occur in the electrophoresis but not detected in the conditions used.

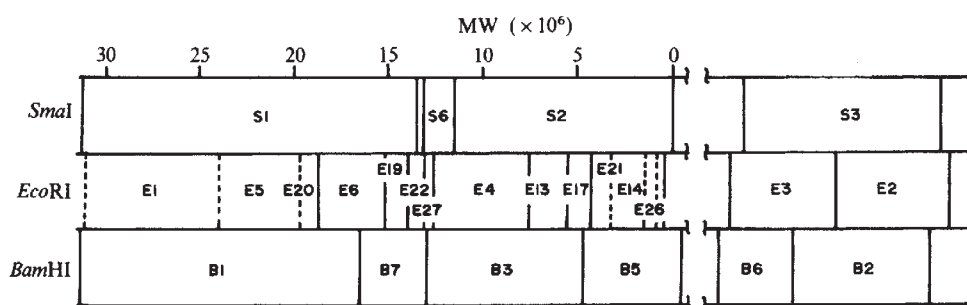


Fig. 3 A map of restriction enzyme cleavage sites near the origin of replication. Relative location of the fragments in Fig. 1 and Table 1 were determined as discussed in the text. To indicate the MW of the fragments, the right hand edge of the *Sma*I-2 fragment was taken as a reference point.

proteins and RNA<sup>3,4</sup>. Asymmetric location of the DNA fragments in the S-complex at one side of the replication origin was assumed by comparing restriction enzyme fragments of this DNA with those of the pulse-labelled DNA at the region of the origin of replication<sup>4,5</sup>. The present mapping data are consistent with the asymmetric location of the S-complex. Judging from the size of the fragments, there are homologues of the E1 and E5 fragments in the DNA from the S-complex, while no fragments to the right hand side of E20 have homologues in the complex DNA. This result is also consistent with the genetic evidence for the asymmetric association of the origin region of the *B. subtilis* chromosome with the cell membrane<sup>3,4</sup>.

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## Effect of novobiocin on initiation of DNA replication in *Bacillus subtilis*

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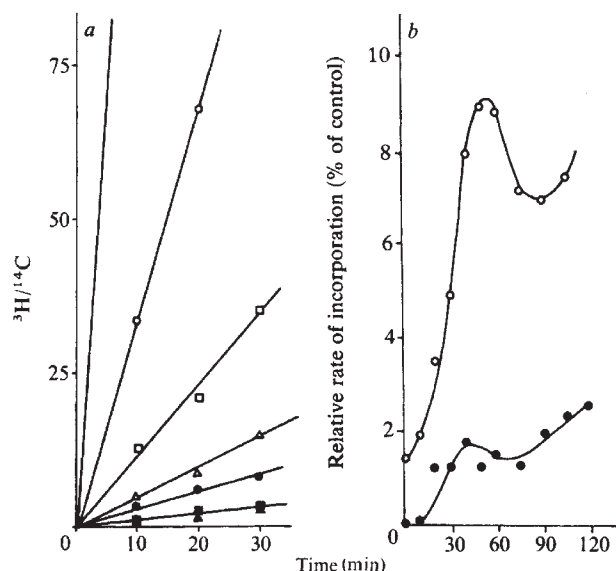
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The initiation of DNA replication of small replicons *in vitro* involves conformational changes in the whole DNA molecule or in the region near to the replication origin<sup>1-4</sup>. One striking finding has been the role of DNA gyrase (that is, the necessity for supercoiled structure) in the initial stage of ColE1 replication *in vitro*<sup>5,6</sup>. However, little is known about the effect of gyrase on the initiation of replication of bacterial chromosomes *in vivo*. We have constructed a map of cleavage sites of restriction enzymes at the region of the origin of replication of the *Bacillus subtilis* chromosome (accompanying paper<sup>7</sup>). This has now enabled us to examine the effect of novobiocin, a selective inhibitor of DNA gyrase<sup>8</sup>, on the replication of the specific chromosomal segments near the origin and to seek a possible role for the gyrase in the initiation of chromosomal replication. We have found that only a limited segment of the chromosome at the origin region was replicated in the presence of novobiocin. This effect allowed us to locate the site of the origin of replication to within a DNA fragment of molecular weight  $3.4 \times 10^6$ .

We used a spore germination of a thymine-requiring mutant of *B. subtilis*, CRK2000 (*leu*, *trp*, *thy*)<sup>9</sup>, to synchronise the initiation of replication. The mutant spores were germinated in a synthetic medium, GM11 (ref. 10), in the absence of thymine for 150 min; <sup>3</sup>H-thymidine (dThd) was then added to initiate replication synchronously.

Figure 1 shows that replication started immediately after the addition of dThd. Therefore, the region which was labelled early probably contained the region of the origin of replication. When novobiocin was added at various concentrations 60 min before dThd, DNA replication was markedly inhibited, in a manner dependent on the inhibitors concentration. At each concentration replication proceeded linearly at reduced rates. When novobiocin was added at various times during the thymine-free germination and thymidine added later at the 150th min to all samples, varied effects of the drug were observed. Figure 1b shows that the initiation of replication was further reduced when novobiocin was added during the first 45–50 min after the onset of germination. After 45–50 min the reduced rates became fairly constant, depending only on the novobiocin concentration. As DNA replication was initiated synchronously at the 45–50th min during germination in the presence of thymidine, the initiation potential (the cell's ability to initiate replication) was assumed to be formed during the first 45 min. Data in Fig. 1b suggest an inhibitory effect of novobiocin on the formation of the initiation potential during germination.

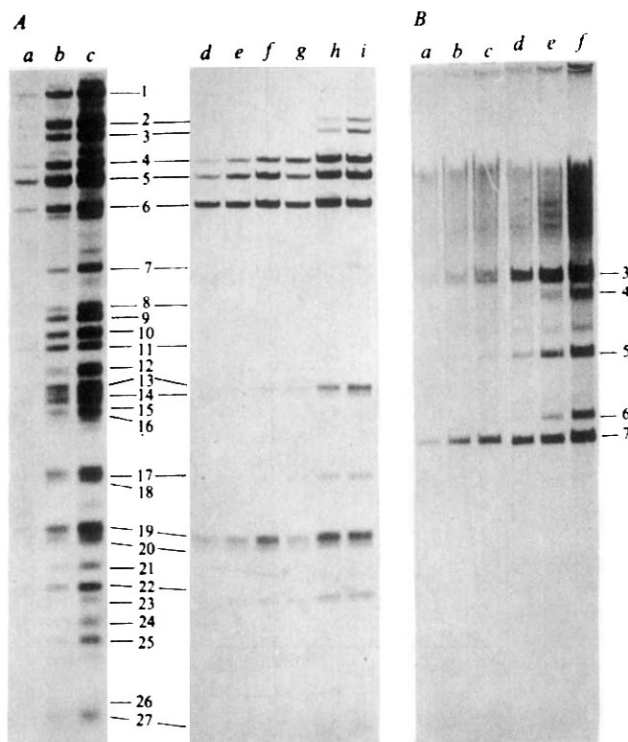
To examine the nature of this reduced replication in the presence of novobiocin, chromosomal segments which were replicated in the presence of novobiocin were identified by digesting <sup>3</sup>H-labelled DNA with restriction endonucleases and then analysing the resulting fragments by agarose gel electrophoresis and fluorography. Figure 2 shows that only a limited number of *EcoRI* fragments were labelled when  $10 \mu\text{g ml}^{-1}$  novobiocin was used. The number of labelled fragments increased more slowly with the lower concentration,  $5 \mu\text{g ml}^{-1}$ . The quantitative measurement of the label in each fragment is shown in Fig. 3A, which indicates that fragments were labelled in a definite order in the presence of novobiocin. Thus, fragment E19 was the first to be labelled, followed by fragments E22, E27, E6, E20, E5 and so on. Identical results were obtained with *Bam*HI fragments. Figure 2 also shows that fragment B7, which



**Fig. 1** The initiation of DNA replication in the presence of novobiocin. *a*, spores of *B. subtilis* CRK2000 (*leu*, *trp*, *thy*)<sup>9</sup> were labelled with <sup>14</sup>C-thymine as reported previously<sup>9</sup>. The labelled spores were germinated in a synthetic medium GM11 (ref. 10), in the absence of thymine after heat activation at 80 °C for 15 min. After 90 min, novobiocin (Boehringer Mannheim) was added at various concentrations and at the 150th min <sup>3</sup>H-dThd (Radiochemical Centre, Amersham), 5 μCi per 75 ng per ml, was added to initiate DNA replication. Thereafter, 1.0-ml samples were taken at the times indicated and precipitated in 10% trichloroacetic acid after incubation in 0.2 M KOH for 30 min at 80 °C. The precipitates were collected on a glass fibre filter and radioactivity were measured in a Beckman scintillation counter LS7000. Incorporation of <sup>3</sup>H-dThd was normalised by <sup>14</sup>C-activity in the spore. —, No drug; —○—, 2.5 μg ml<sup>-1</sup>; —□—, 5 μg ml<sup>-1</sup>; —△—, 7.5 μg ml<sup>-1</sup>; —●—, 10 μg ml<sup>-1</sup>; —■—, 20 μg ml<sup>-1</sup>; —▲—, 40 μg ml<sup>-1</sup> novobiocin. *b*, The <sup>14</sup>C-labelled spores were germinated as in *a* except that novobiocin was added at various times during the thymine-free germination. <sup>3</sup>H-dThd, 5 μCi per 75 ng per ml, was added to all samples at the 150th min. The rate of incorporation was measured for each sample from the slope of linear incorporation as in *a*. It was then normalised by the initial rate of the control sample and plotted against the time of novobiocin addition. —○—, 5 μg ml<sup>-1</sup>; —●—, 10 μg ml<sup>-1</sup> novobiocin.

contained fragment E19, was replicated first, followed by fragments B3 and B5.

Comparing these results with the map of the restriction sites near the origin of replication (see accompanying paper), we estimated time courses of initiation and replication in the presence and absence of novobiocin. The results demonstrated a bidirectional replication starting within fragment B7. Furthermore, it shows clearly that only a limited region around the origin was replicated in the presence of  $10 \mu\text{g ml}^{-1}$  novobiocin. The remarkably high level of incorporation into fragments E19 and E22 was not due to a premature re-initiation, because chloramphenicol had no effect on this level when added 60 min before the isotope. This indicates that some cells replicated to lengths of only  $1.0\text{--}1.5 \times 10^6$  MW in the presence of the drug. When germinating spores were labelled for short times in the absence of novobiocin, the labelling patterns obtained were essentially the same as those with novobiocin. However, the rate of elongation was too fast to determine the labelling sequence among early replicating fragments other than fragments E19, E22 and E27 (Fig. 3). The unusually high level of labelling in fragment E5 might have been due to its contamination by one or two other fragments which were not replicated in the presence of novobiocin. This is further suggested by the finding that a part of fragment E5 from the control sample was digested by *Bam*HI, whereas fragment E5 from the novobiocin sample had no *Bam*HI site. Also, in certain conditions, the control fragment E5 split into two or more bands.

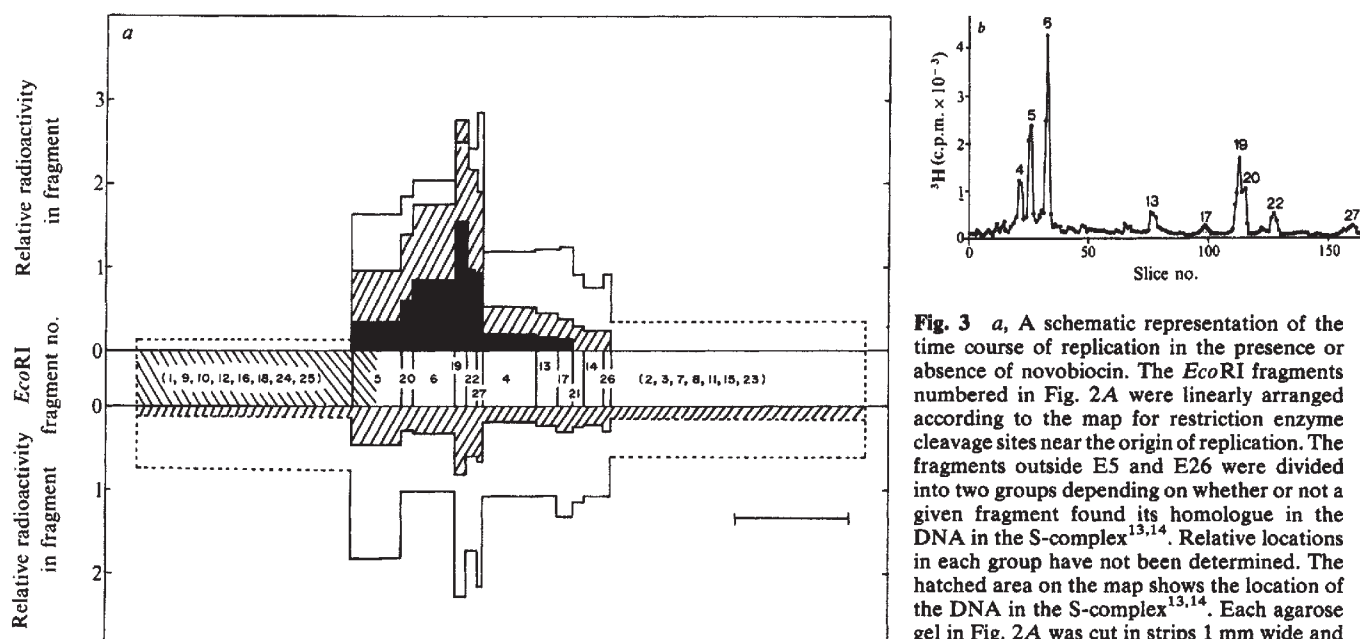


**Fig. 2** Analysis by restriction endonucleases of DNA, pulse labelled during the initiation of replication in the presence of novobiocin. Spores were germinated as in Fig. 1. Novobiocin and  $^3\text{H}$ -dThd, 10  $\mu\text{Ci}$  per 50 ml, were added at the 90th and 150th min, respectively. The labelling was stopped by pouring the culture into an equal volume of ethanol-phenol (75% ethanol, 2% phenol in 21 mM Na-acetate buffer, pH 5.1, and 2 mM EDTA). Cells were collected by centrifugation lysed with 1 mg ml $^{-1}$  lysozyme in a lysis buffer (20 mM Tris-HCl, pH 8.1, 100 mM KCl, 1 mM EDTA) for 30 min and then treated with 1% Sarkosyl (Geigy) and 500  $\mu\text{g}$  ml $^{-1}$  pronase E (Kaken Kagaku). DNA was purified by repeating the phenol extraction and precipitation by ethanol in the lysis buffer. The purified DNA was digested with *Eco*RI (Miles) or *Bam*HI (prepared as reported previously<sup>11</sup>) using standard methods, and subjected to electrophoresis in 0.7% agarose gel and detected by fluorography as described by Marsh and Worcel<sup>12</sup>. **A**, The patterns for *Eco*RI digests. *a*-*c* Are DNA samples labelled for 45, 90 and 135 s, respectively, without the drug. *d*-*f* Are DNA samples labelled for 10, 20 and 30 min with 10  $\mu\text{g}$  ml $^{-1}$  novobiocin. *g*-*i* Are DNA samples labelled for 10, 20 and 30 min with 5  $\mu\text{g}$  ml $^{-1}$  novobiocin. **B**, The patterns for *Bam*HI digests. *a*-*c* Are samples labelled for 5, 10 and 15 min with 10  $\mu\text{g}$  ml $^{-1}$  novobiocin. *d*-*f* Are samples labelled for 5, 10 and 15 min with 5  $\mu\text{g}$  ml $^{-1}$  novobiocin.

The experimental results presented here indicate that novobiocin had two types of effect on DNA replication. First, it inhibited formation of the cell's potential ability to initiate DNA replication. This may be a direct effect on DNA structure or it may act indirectly through inhibition of RNA synthesis. Second, it permitted a limited amount of replication at a specific segment of the chromosome near the origin of replication, once the initiation potential had been formed in the cell.

These results suggest that the conformational changes in the chromosome, due in part to the function of DNA gyrase, is essential both for initiation and for further elongation even after the proper conformation has been formed in the cell. These effects of novobiocin on chromosomal replication resemble those of gyrase on *in vitro* replication of CoIE1 plasmid<sup>5,6</sup>.

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**Fig. 3** **a**, A schematic representation of the time course of replication in the presence or absence of novobiocin. The *Eco*RI fragments numbered in Fig. 2A were linearly arranged according to the map for restriction enzyme cleavage sites near the origin of replication. The fragments outside E5 and E26 were divided into two groups depending on whether or not a given fragment found its homologue in the DNA in the S-complex<sup>13,14</sup>. Relative locations in each group have not been determined. The hatched area on the map shows the location of the DNA in the S-complex<sup>13,14</sup>. Each agarose gel in Fig. 2A was cut in strips 1 mm wide and put in a scintillation mixture to measure  $^3\text{H}$

activity. The result obtained for Fig. 2A-f is shown in **b** as an example. The relative radioactivity under the area of each fragment peak was normalised by its MW and then plotted against the map position. The results with novobiocin were drawn above the fragment map. The shaded area represents the replication for 10 min in the presence of 10  $\mu\text{g}$  ml $^{-1}$  novobiocin (Fig. 2A-d). The hatched area represents the replication for 30 min with 10  $\mu\text{g}$  ml $^{-1}$  novobiocin (Fig. 2A-f). The open area represents the replication for 30 min with 5  $\mu\text{g}$  ml $^{-1}$  novobiocin (Fig. 2A-i). Control samples are shown below the map. The hatched area represents the replication for 45 s (Fig. 2A-a) and the open area replication for 90 s (Fig. 2A(b) both in the absence of novobiocin. The dotted line shows the average radioactivities within the group of fragments shown in parentheses.



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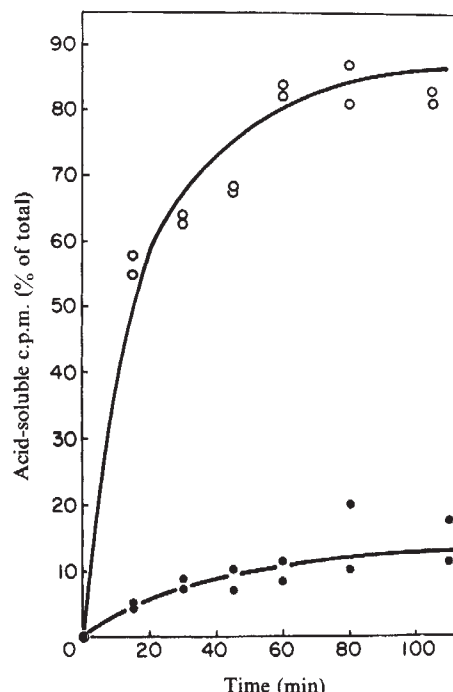
## Ribonucleotides in DNA newly synthesised in 3T6 cells *in vivo*

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Within the field of DNA replication, considerable interest has focused in recent years on the mechanism of initiation of synthesis of DNA molecules. *In vitro* replication systems from *Escherichia coli* have been instrumental in uncovering a priming function for ribonucleotides on the earliest intermediates of DNA polymerisation *in vitro* and in identifying the proteins involved<sup>1-3</sup>. *In vitro* replication systems from mammalian cells that permit the use of the phosphate-transfer method for detection of RNA-DNA junctions as well as direct labelling of the RNA moiety of the molecules have suggested a similar role for ribonucleotides in DNA synthesis in eukaryotes<sup>4-6</sup>. However, the existence of this mechanism in mammalian cells *in vivo* has not been established. Here we report the first evidence that a significant proportion of the earliest intermediates in mammalian DNA polymerisation *in vivo* do, in fact, possess ribonucleotides, presumably because their synthesis was initiated with one or more ribonucleotides.

In *E. coli* several types of experiment have been carried out with nascent DNA from intact cells. The direct approach uses purification of nascent DNA molecules, labelling of their 5'-ends with <sup>32</sup>P using polynucleotide kinase, and subsequent demonstration of the release of 2'(3'), 5'-[<sup>32</sup>P]-ribonucleoside diphosphates on alkaline hydrolysis<sup>9</sup>. We used this approach to study DNA synthesis in mammalian cells, and found that, in addition to difficulties due to the presence of contaminating RNA molecules not covalently attached to DNA, the presence of a large number of small non-nascent DNA molecules of uncertain origin obscured an adequate demonstration of ribonucleotides at the 5'-ends of nascent molecules<sup>10,11</sup>. An alternative approach involves alkaline hydrolysis of nascent DNA molecules phosphorylated at the 5'-end with polynucleotide kinase. The appearance of 5'-OH termini, on alkaline hydrolysis of the nascent polynucleotide is assayed by labelling with <sup>32</sup>P using polynucleotide kinase<sup>9,12</sup>, or by digestion of the molecules with the 5'-OH-specific exonuclease, spleen phosphodiesterase<sup>9,13</sup>. In *E. coli*, there is good evidence that some of the nascent DNA molecules are initiated with ribonucleotides *in vivo*. *Physarum* is the only eukaryote in which evidence (by phosphate transfer) for RNA priming *in vivo* has been obtained<sup>14</sup>. Earlier experiments using differences in buoyant density as a measure of covalent linkage of RNA to nascent DNA<sup>15,16</sup> have been shown to be subject to artefact<sup>17</sup>. We have examined nascent DNA molecules synthesised in intact murine 3T6 cells for the presence of ribonucleotides by the method which uses spleen exonuclease.



**Fig. 1** Time course of digestion of DNA by spleen exonuclease. DNA uniformly labelled with <sup>3</sup>H-dThd and extracted from 3T6 cells with SDS was deproteinised by phenol-chloroform extraction. Heat-denatured DNA was separated from RNA by chromatography on nitrocellulose<sup>10</sup>. The material was then sonicated extensively and fractionated on a neutral sucrose gradient. Fractions containing DNA 1,200-2,400 nucleotides long were pooled. After re-precipitation with isopropanol the DNA (300 nmol nucleotide per ml in 50 mM Tris-Cl, pH 8) was heat denatured. One half was incubated with bacterial alkaline phosphatase purified as previously described<sup>9</sup> at a concentration of 1 unit ml<sup>-1</sup> at 65 °C for 1 h. The other half was exposed to T4 polynucleotide kinase (purified as previously described<sup>9</sup>) and ATP in 50 mM Tris-Cl (pH 8.1), 2 mM potassium phosphate, 10 mM MgCl<sub>2</sub> and 20 mM 2-mercaptoethanol for 60 min at 25 °C. The amount of ATP required in the reaction mixture was determined by estimating the number of 5'-ends from the concentration of nucleotide and the size of the DNA. ATP was added in a 500-fold molar excess over the number of 5'-ends in the sample. Both kinase and phosphatase-treated portions were then made 20 mM in EDTA, 100 mM in NaCl and 30 µg ml<sup>-1</sup> in SDS extracted once with phenol-chloroform (1:1) and precipitated with isopropanol. DNA redissolved in water was adjusted to the following conditions: 150 mM CH<sub>3</sub>COONa (pH 5.5), 10 mM EDTA, 50 mM Na<sub>2</sub>SO<sub>4</sub>, 30-100 µg ml<sup>-1</sup> DNA. The DNA was heated at 100 °C for 20 s. Spleen exonuclease, purified as described by Bernardi and Bernardi<sup>18</sup>, was then added to a concentration of 33 µg ml<sup>-1</sup> and incubated with the DNA at 45 °C for the time indicated. Each incubation, in duplicate, was then made 400 µg ml<sup>-1</sup> in salmon sperm DNA and 10% in trichloroacetic acid. After 20 min at 4 °C, it was centrifuged at 16,000g for 10 min. The precipitate was washed once with 10% trichloroacetic acid and redissolved in 0.2 M NaOH at 100 °C. Radioactivity in the supernatant and precipitate was determined in an aqueous scintillation counting cocktail after neutralisation. O, Phosphatase-treated DNA; ●, kinase-treated DNA.

Spleen exonuclease was purified from porcine spleen by the procedure described by Bernardi and Bernardi<sup>18</sup>. The specificity of the enzyme for 5'-OH-terminated DNA is shown in Fig. 1. The maximal extent of digestion of 5'-OH DNA was 85-90%. The enzyme shows a marked preference for single-stranded molecules<sup>18</sup> and the residual resistance is probably due to the presence of secondary structure in the DNA. In these conditions 5'-phosphoryl DNA was degraded to the extent of about 15%. We suspect that this is the result of the presence of small amounts of endonuclease in the exonuclease preparation<sup>19,20</sup>. About 10% of circular single-stranded bacteriophage ΦX174 DNA was degraded to mononucleotides by the enzyme in 60 min (1 µg ml<sup>-1</sup> <sup>32</sup>P-labelled ΦX DNA + 100 µg ml<sup>-1</sup> denatured salmon sperm DNA + 33 µg ml<sup>-1</sup> enzyme; other conditions as in Fig. 1). The 3T6 cell DNA preparations were not contaminated by endonuclease activity as the rate of digestion by spleen exonuclease of phosphatase- or kinase-treated DNA (Fig. 1) was unchanged by preincubation of the DNA for 8 h at 37 °C or for 1 h at 45 °C (data not shown). Other experiments

**Table 1** Per cent digestion by spleen exonuclease of pulse-labelled and pulse-chase-labelled DNA after alkali or phosphatase treatment

| DNA                                | Size class | Treatment before spleen exonuclease |           |             |
|------------------------------------|------------|-------------------------------------|-----------|-------------|
|                                    |            | None                                | Alkali    | Phosphatase |
| <sup>3</sup> H-Pulse               | a          | 9.5                                 | 25        | 50          |
| <sup>3</sup> H-Pulse-chase         | a          | 5.0                                 | 5.0       | 38          |
| <sup>32</sup> P-Uniformly labelled | a          | 8.8 ± 0.3                           | 4.7 ± 0.2 | 43 ± 0.3    |
| <sup>3</sup> H-Pulse               | b          | 8.8                                 | 23        | 53          |
| <sup>3</sup> H-Pulse-chase         | b          | 4.5                                 | 4.3       | 43          |
| <sup>32</sup> P-Uniformly labelled | b          | 5.0 ± 0.5                           | 3.7 ± 0.2 | 44 ± 1      |
| <sup>3</sup> H-Pulse               | c          | 18                                  | 36        | 78          |
| <sup>3</sup> H-Pulse-chase         | c          | 8.5                                 | 5.8       | 75          |
| <sup>32</sup> P-Uniformly labelled | c          | 7.3 ± 0.2                           | 5.0 ± 0.8 | 69 ± 1      |

<sup>3</sup>H-DNA, labelled briefly (pulse) or (see text) for several hours (pulse-chase), was mixed with control <sup>32</sup>P-DNA of the appropriate size as indicated in Fig. 2 legend and treated with polynucleotide kinase and ATP in the conditions described in Fig. 1 legend. Each sample was then divided into three portions. One portion was adjusted to 0.4 M NaOH and all three were incubated at 37 °C for 8 h. The alkaline portion was neutralised and one of the two portions not exposed to alkali was treated with phosphatase. DNA in all three samples was extracted with phenol-chloroform as described in Fig. 1 legend and precipitated. All samples were then incubated with spleen exonuclease for 20 min in the conditions described in Fig. 1 legend. Each determination of acid-soluble and acid-insoluble radioactivity involved 5–50 × 10<sup>3</sup> c.p.m. each of <sup>32</sup>P and <sup>3</sup>H. The values for digestion of <sup>32</sup>P-uniformly labelled DNA accompanying the two <sup>3</sup>H-labelled preparations (pulse and pulse-chase) have been averaged and the standard error is indicated.

(not shown) indicated that the extent of digestion of DNA varied with the enzyme concentration, reaching a plateau at 25 µg ml<sup>-1</sup>. The rate of digestion was tested at different substrate concentrations and was found to be constant in the range 30–200 µg ml<sup>-1</sup> of DNA. Thus, all digestions with spleen exonuclease were carried out for 20 min at 45 °C with 33 µg ml<sup>-1</sup> of enzyme and 30–100 µg ml<sup>-1</sup> of substrate.

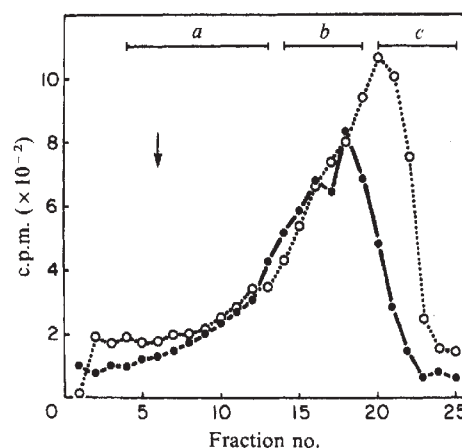
DNA synthesised by cultures of 3T6 cells was labelled with <sup>3</sup>H-thymidine (<sup>3</sup>H-dThd) for 1 min at 37 °C after 5 min at 4 °C to allow equilibration of intracellular precursor pools. In some cultures DNA synthesis was abruptly stopped by lysis of the cells with SDS at 100 °C (ref. 10) whereas other cultures were incubated in the presence of unlabelled dThd for an additional 2 h. All lysates were briefly sonicated to fragment the high molecular weight DNA. The preparation was deproteinised by treatment with Pronase and extraction with phenol-chloroform and RNA was separated from DNA by chromatography on nitrocellulose. Sonicated <sup>32</sup>P-labelled 3T6 cell DNA was added to the <sup>3</sup>H-dThd-labelled preparations to serve as an internal control for all subsequent analyses. The DNA was fractionated into three classes by centrifugation in neutral sucrose density gradients, as shown for the pulse-labelled DNA in Fig. 2. The size of the DNA in pools a, b and c estimated from the sedimentation constants was 4–15, 0.2–4 and <0.2 kilobases respectively. This DNA was then reacted with polynucleotide kinase and enough ATP to give a 500-fold molar excess of ATP over the number of 5'-ends in the sample. Aliquots of the preparation were subjected to alkaline hydrolysis or alkaline phosphatase treatment. The DNA was then tested for spleen exonuclease sensitivity as described above. Immediately before each step in this purification and before all enzyme treatments, the DNA was heated to 100 °C for 20 s. The results of a typical experiment are shown in Table 1.

Treatment of DNA with alkali results in a significantly increased sensitivity to spleen exonuclease digestion in pulse-labelled DNA that is absent in pulse-chase-labelled DNA and in the internal control. We interpret these data to mean that nascent DNA molecules contain ribonucleotides. However, spleen exonuclease sensitivity after alkali treatment alone is indicative of an alkali-labile structure in the molecule, not necessarily a ribonucleotide. The results of four additional experiments on separate preparations of pulse-labelled DNA are summarised in Table 2.

The background levels of spleen exonuclease sensitivity in these experiments are slightly higher than those for the experi-

ment shown in Table 1. The background level of digestion of <sup>3</sup>H-pulse-labelled untreated DNA averaged 30%. Treatment with either alkali or ribonuclease before spleen exonuclease increased this to an average of 40%. The greatest increase (18%) was observed with the smallest molecules (size class c), the least increase (3%) with the largest molecules (size class a). The background digestion of uniformly <sup>32</sup>P-labelled DNA averaged 16%. Alkali or ribonuclease treatment increased this average by only 2%. The fact that ribonuclease acts in a manner quantitatively identical to alkali strongly suggests that the increase in sensitivity is due to the presence of ribonucleotides.

Not all the molecules contained ribonucleotides. The maximum extent of spleen exonuclease sensitivity in a 20-min incubation with the enzyme is demonstrated by the samples treated with phosphatase. The resistance to digestion by spleen exonuclease increases with the size of the molecules, probably because the likelihood of the enzyme encountering secondary structure in the DNA also increases. The increase in sensitivity due to alkali or ribonuclease as a proportion of the increase due to phosphatase allows an estimation of the fraction of nascent molecules that possess ribonucleotides. In this manner we estimate from Table 2 that 13%, 27% and 42% of the molecules in size classes a, b and c, respectively, contain ribonucleotides. Thus, the presence of ribonucleotides on the smallest, and presumably most nascent, molecules extends the observations in Table 1 that pulse-labelled, but not pulse-labelled and chased, molecules seemed in part to contain ribonucleotides. Knowing the size of the molecules and assuming that the <sup>3</sup>H is uniformly distributed within them, we estimate that 96% of the molecules are in class c, so that about 40% of all nascent molecules possess ribonucleotides. This agrees reasonably well with previous studies by other methods on the proportion of Okazaki pieces



**Fig. 2** Neutral sucrose gradient fractionation of <sup>3</sup>H-pulse-labelled DNA and uniformly <sup>32</sup>P-labelled DNA. 3T6 cells grown to confluence in 5% serum were partially synchronised by replating in 10% serum 24 h before labelling<sup>24</sup>. Cell cultures in plates were floated in an ice bath at 4 °C and exposed to 500 µCi ml<sup>-1</sup> (60 Ci mmol<sup>-1</sup>) <sup>3</sup>H-dThd in culture medium at 4 °C for 5 min. The plate was then floated in a 37 °C water bath and an equal volume of culture medium at 37 °C containing 500 µCi ml<sup>-1</sup> <sup>3</sup>H-dThd was added to the plate. We find that this method of pre-equilibration with dThd<sup>25</sup> results in the incorporation of two to five times more <sup>3</sup>H-dThd during the period at 37 °C. After 1 min at 37 °C DNA replication was rapidly terminated by lysing the cells with 1% SDS at 100 °C for 1 min<sup>10</sup>. The lysates from 2–4 × 10<sup>7</sup> cells were pooled and sonicated briefly to reduce the size of the high molecular weight DNA. The lysate was then treated with Pronase-B (1 mg ml<sup>-1</sup>, 37 °C, 2.5 h in the presence of 0.4 µg ml<sup>-1</sup> polyvinyl sulphate), extracted twice with phenol-chloroform (1:1) and precipitated with isopropanol. Over 90% of the RNA was removed by chromatography on nitrocellulose<sup>10</sup>. Uniformly <sup>32</sup>P-labelled DNA was prepared by growing 3T6 cells for 2 d in phosphate-free medium containing 10 µCi ml<sup>-1</sup> <sup>32</sup>P-ortho-phosphate. It was purified as described above using sonication to reduce its size. Nascent <sup>3</sup>H-DNA and total <sup>32</sup>P-DNA were fractionated together after brief denaturation on 5–20% sucrose gradients in 1 M NaCl, 1 mM EDTA and 50 mM Tris-Cl, pH 8, in a SW56 rotor at 50,000 r.p.m. for 3.5 h at 20 °C. Fractions were collected from the bottom, assayed for radioactivity and pooled as indicated. The arrow denotes the position of single-stranded linear ΦX174 DNA (24S). ○, <sup>3</sup>H-pulse-labelled DNA; ●, <sup>32</sup>P-total DNA.



**Table 2** Per cent digestion by spleen exonuclease of pulse-labelled DNA after alkali, ribonuclease or phosphatase treatment

| DNA                                                | Size class | Treatment before spleen exonuclease |        |              |             |
|----------------------------------------------------|------------|-------------------------------------|--------|--------------|-------------|
|                                                    |            | None                                | Alkali | Ribonuclease | Phosphatase |
| <sup>3</sup> H-DNA (nascent)                       | a          | 35                                  | 37     | 39           | 59          |
|                                                    | b          | 28 ± 3                              | 36 ± 3 | 38 ± 3       | 61          |
|                                                    | c          | 28 ± 4                              | 46 ± 2 | 46 ± 2       | 71 ± 9      |
| <sup>32</sup> P-DNA (sonicated uniformly labelled) | a          | 24                                  | 23     | 21           | 60          |
|                                                    | b          | 15 ± 2                              | 19 ± 4 | 20 ± 3       | 59          |
|                                                    | c          | 10 ± 3                              | 12 ± 5 | 16 ± 3       | 69 ± 9      |

Cultures of 3T6 cells were pulse-labelled with <sup>3</sup>H-dThd as described for Fig. 2. After treatment with Pronase for 1 h the lysate was heated at 100 °C for 4 min, chilled and centrifuged for 45 min at 45,000 r.p.m. in a Ti60 rotor at 4 °C to separate high and low molecular weight DNA. The supernatant contains 0.1% of the total DNA and 75–80% of the incorporated radioactivity<sup>10</sup>. DNA in the supernatant was then purified as described for Fig. 2 without sonication, mixed with sonicated <sup>32</sup>P-DNA and fractionated into the three size classes on neutral sucrose gradients. Further analyses were carried out as described for Table 1. Ribonuclease treatment used ribonuclease A (Worthington), 20 µg ml<sup>-1</sup>, plus ribonuclease T1 (Worthington), 20 units ml<sup>-1</sup> (heated at 80 °C for 10 min to inactivate DNases), for 30 min at 37 °C. Each determination of acid-soluble and acid-insoluble radioactivity involved 5–50 × 10<sup>3</sup> c.p.m. each of <sup>32</sup>P and <sup>3</sup>H. The values given for the b and c size classes are the average of four experiments on four separate preparations of <sup>3</sup>H and <sup>32</sup>P DNA; the s.e.m. is indicated. When no standard error is shown only one experiment was carried out. Degradation of control 5'-phosphorylated DNA may result from contaminating endonucleolytic activity<sup>19,20</sup>; our observations that 10–15% of circular ΦX DNA is degraded support this possibility. The enzyme concentration, size and type of DNA, conditions of digestion, and techniques used to measure hydrolysis affect the results. In some of our experiments, especially this Table, 5'-phosphorylated DNA was degraded more than the usual 10–15%. We think this is because the <sup>32</sup>P-labelled DNA was a few weeks old and that radiolysis had enhanced its sensitivity to alkali or enzymes. The data in Table 1 were obtained with freshly prepared <sup>32</sup>P-DNA. The <sup>3</sup>H-DNA was always used as soon as practicable after its preparation and we have no reason to think that radiolysis was contributing significantly to its sensitivity to spleen exonuclease. We have found that fragmentation of the DNA by either sonication or partial digestion with DNase I yielded preparations which showed similar low (or high, after phosphatase treatment) sensitivities to spleen exonuclease.

possessing oligoribonucleotide primers which place the value at 33–50% (refs 8, 21).

We routinely observed that nascent DNA not treated with alkali or RNase was more sensitive to spleen exonuclease digestion than was non-nascent DNA, particularly among the smallest molecules after correction for the degradation of the control <sup>32</sup>P-DNA. This might be due to the presence of structures in some nascent molecules that are sensitive to an endonuclease activity in the exonuclease preparation (for example, apyrimidinic sites of uracil excision).

The existence of RNA priming of DNA synthesis has been regarded with caution because of the difficulty in demonstrating this mechanism *in vivo*<sup>22</sup>. This is especially true of mammalian DNA, where such data have been lacking. The above experiments show that it is possible to detect this mechanism *in vivo* in intact mammalian cells by using different methods from those used in the analysis of the products of synthesis *in vitro*.

While this work was undergoing review Tseng *et al.*<sup>23</sup> reported results showing good evidence for the presence of short oligoribonucleotides on the 5'-ends of nascent DNA molecules synthesised *in vivo* in human lymphoblasts.

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## Biological degradation of TCDD in rats

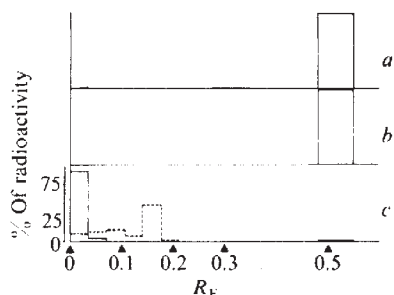
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Apart from its high toxicity<sup>1,2</sup>, the chemical stability and persistence of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the environment bring about additional hazards<sup>3,4</sup>. However, until now there has been no evidence for degradation of this dioxin in biological systems, and it is considered unusual that a compound which is insusceptible to biological attack should be so extremely toxic. In *in vitro* studies<sup>5</sup> with microsomal preparations from mouse, rat and rabbit liver, as well as *in vivo*, no metabolite of TCDD has been detected by several workers<sup>6–8</sup>. Recent data, showing a lower toxicity of TCDD in rats after stimulation of the hepatic mixed-function oxidase, suggested possible metabolic transformation of this compound<sup>9</sup>. In the present study, we used tritiated TCDD to search for metabolites in the bile of rats and found marked changes in lipophilicity and mobility in TLC of the excreted radioactivity. Treatment with diazomethane yielded at least two new compounds. These observations hinted at the formation of phenolic hydroxyl groups in the dioxin molecule.

Commercially available tritium-labelled TCDD with a specific activity of 41 Ci mmol<sup>-1</sup> was purified by preparative gaschromatography using consecutively two stationary phases with different polarity (OV 101, DC 560); a radiochemical purity higher than 98.7% was thus obtained. The test animals were female rats from the Sprague-Dawley-derived ZUR:SV-Z strain, weighing about 260 g. The bile duct was cannulated in both directions with polyethylene catheters which then led to the neck of the animal. After a recovery period of a few days the rats received oral doses of about 20 µCi TCDD dissolved in corn oil and were placed in suitable restriction cages allowing free access to water and feed. Bile was collected in a fraction collector for 3–4 days and then the animals were killed.

The concentration of radioactivity in the bile remained relatively constant throughout the whole experimental period, about 1% of the dose being excreted daily. In the liver tissue, 8.5–12% of the administered radioactivity was found. This radioactivity could be extracted almost completely using dichloromethane as solvent, with about 2% remaining in the residue. In contrast, only 1.5% of the radioactivity from the bile was extractable. This radioactivity was dialysable, so that binding to proteins or other macromolecules could be excluded. However, after incubation of the bile (or the dialysate) with glucuronidase/arylsulphatase (Boehringer), 75% of the radioactivity moved into the organic phase. Only a small part could be removed by lyophilisation (5% from bile, 0.2% from liver). This fraction most probably represents tritiated water because the specific activity remained constant in several successive fractions of the distillate and was not extractable with hexane.



**Fig. 1** Radio-TLC of: *a*, tritiated TCDD (as received, not purified); *b*, liver extract; *c*, bile extract (after enzymatic treatment). Precoated silica-gel 60 sheets (Merck), with dichloromethane as developing solvent, were used. The dotted lines show distribution of radioactivity after methylation with diazomethane. Levels of radioactivity were determined in scrapings of 0.5–1 cm zones and are expressed as per cent of the total amount of radioactivity on the silica-gel sheet.

In TLC, radioactivity extracted from the liver was localised at an  $R_F$  value of 0.52, where TCDD was also found (Fig. 1). The compound extracted from bile after the enzymatic treatment behaved quite differently. Most of the activity was concentrated at the starting zone, demonstrating a more polar character, with only a small fraction, 1.5%, located at  $R_F$  0.55. Gaschromatographic examination of this extract on a SE 30 column revealed a retention time of the radioactivity similar to that of TCDD; TLC mobility of the material trapped during the GLC examination remained unchanged. When labelled TCDD was added to bile and the mixture left to stand for a few hours, this added fraction could always be extracted with dichloromethane and was found at the position of TCDD on thin layer chromatograms. Thus, chelation of TCDD by any constituents of the bile was excluded.

Treatment of bile extracts with diazomethane led to the formation of at least two new compounds with lower polarity than TCDD, exhibiting  $R_F$  values of about 0.09 and 0.16.

As only small amounts of the compound were available—concentration in the bile fluid was 50–100 pg ml<sup>-1</sup>—attempts at mass spectrum analysis were unsuccessful.

Thus, the present results indicate that TCDD is excreted into bile in a metabolised form, as a water-soluble conjugate. These conjugates can be cleaved by enzymatic treatment yielding relatively polar compounds with low mobility on thin layer chromatograms. Esterification with diazomethane diminishes polarity and consequently mobility of the derivative on TLC is increased. Most probably, conjugation occurs with phenolic hydroxyl groups, but their position and number are unknown. Preliminary experiments with double-labelled <sup>14</sup>C/<sup>3</sup>H-TCDD showed no change of the ratio of the two isotopes after metabolism. This observation makes a hydroxylation at the *ortho* positions unlikely. However, cleavage of the ether group also seems feasible. A decision between these possibilities cannot yet be made.

<sup>3</sup>H-TCDD was obtained from A. Kende, Rochester, New York.

*Note added in proof:* Similar results have recently been reported<sup>10</sup>.

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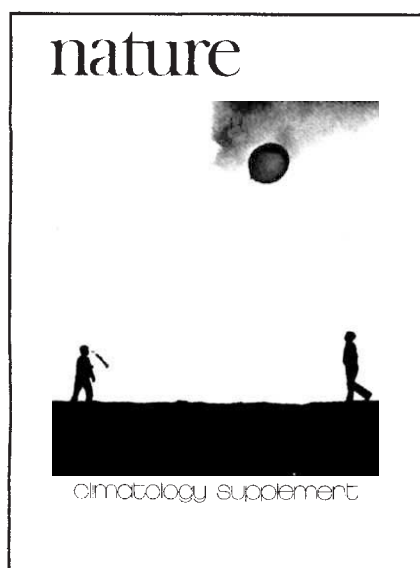
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# matters arising

## Interstellar grains: organic or refractory?

IT has become fashionable to discuss the possibility that interstellar grains may contain significant quantities of organic material (see ref. 1 and refs therein). I draw attention here to new data which preclude the presence of most organic solids as a significant component of the grains in two typical dark clouds. As pointed out by Duley and Williams<sup>2</sup>, virtually all organic compounds containing C and H possess an infrared absorption feature due to C—H stretching at 3.3–3.4  $\mu\text{m}$ . The spectra of several dust-embedded stars in the Ophiuchus and Corona Austrina dark clouds were recently observed with the Anglo-Australian Telescope (Whittet and Blades<sup>3</sup>), in a programme primarily aimed at studying the 3.1  $\mu\text{m}$  ice band. None of these sources, whose visual absorption falls in the range 5–20 mag, shows a feature at 3.3–3.4  $\mu\text{m}$ : the detection limit is an order of magnitude less than the optical depth predicted by Fig. 1 of ref. 2. Similarly (as noted in ref. 2) the spectra of molecular cloud sources with deep ice bands observed by Merrill *et al.*<sup>3</sup> show no C—H feature. It must therefore be concluded that organic grains cannot constitute a significant part of interstellar grain material, even in dense clouds where from other considerations, grain growth is known to occur<sup>5,6</sup>.

It seems that the only model for interstellar grains which can simultaneously satisfy their well established dielectric properties<sup>3,7</sup>, cosmic abundance constraints<sup>8</sup>, and the infrared spectra of highly reddened stars<sup>3,4,9</sup>, is one involving a mixture of refractory particles involving compounds of oxygen—silicates and metal oxides—with a possible addition of water ice in the densest regions. Most of the carbon in interstellar clouds is likely to be tied up in gas-phase CO. Candidate grain materials, such as SiO<sub>2</sub>, FeO and MgO, are, indeed, abundant in the carbonaceous chondrites<sup>10</sup>.

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**SAGAN AND KHARE REPLY**—We have proposed<sup>1</sup> that a complex organic material, called tholins, produced by electrical discharge or ultraviolet (UV) light from mixtures of cosmically abundant gases, is an important candidate material for the interstellar grains (although other material such as ices and silicates must, of course, be present); and that the sputtering and spallation products of tholins can explain the gas phase organic molecules discovered in the interstellar medium by microwave line spectroscopy. As noted in Fig. 3 of our paper<sup>1</sup> and elsewhere<sup>2</sup>, little or no IR absorption is evident in the interstellar medium in the 3.3–3.4  $\mu\text{m}$  region, characteristic of the C—H vibrational transitions of many organic molecules. The observational upper limits correspond, for example, to 10% of carbon in interstellar grains in alkenes and aromatics and 1% in alkanes (ref. 2), and an order of magnitude less according to Whittet. However, as clearly indicated in Table 1 of our paper<sup>1</sup>, the atomic abundance of hydrogen by number in tholins is only a few per cent; and, as is apparent from Fig. 2 of our paper, in most laboratory tholins there is no trace of absorption features in the 3.3–3.5  $\mu\text{m}$  range. We have re-examined various IR spectra of tholins and find no clear C—H stretch peak for raw UV tholins; for UV tholins with elemental sulphur removed; for UV tholins with elemental sulphur removed and the sample heated to 450 °C; for spark tholins; and for spark tholins heated to 650 °C. Tholins have a significantly different structure from graphite. We also re-emphasise that a range of other IR absorption features, reliably identified in the interstellar medium, cannot be understood by refractory materials and ices alone; but have a natural explanation if tholins are a constituent of the interstellar grains. The missing ingredient in the interstellar medium seems to be a complex organic solid which has a very weak C—H absorption feature; tholins seem to satisfy these requirements.

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## The maximum entropy method

THE exposition of the maximum entropy method (MEM) given by Gull and Daniell<sup>1</sup> has been strongly criticised<sup>2</sup> on the basis that it led to some definite solution in situations in which different types of solution are possible (for example, one-peak (one source in astronomical language) and two-peak (double source) solutions). Here we defend Gull and Daniell's theory.

No matter how precise the measurements are, for sufficiently small  $\delta$ , a point source gives the same observational information as do two nearby point sources at distance  $\delta$ . Therefore among all brightness distributions,  $I$ , that are in accordance with observations, there are values of  $I$  corresponding to many-component sources (with as many components as one wishes) with very complicated structure. Astrophysicists want the distribution that contains, crudely speaking, the minimum possible number of components, or, more precisely, the simplest possible distribution in some formal sense. We show here that MEM solves this problem satisfactorily.

How can we formalise this demand? Assuming that complexity is a functional  $\{s(I)$ . We suppose that it can be represented in the form

$$s_0 + \int s_1(\bar{\sigma}, I(\bar{\sigma})) d\bar{\sigma} + \int s_2(\bar{\sigma}_1, \bar{\sigma}_2, I(\bar{\sigma}_2), I(\bar{\sigma}_2)) d\bar{\sigma}_1 d\bar{\sigma}_2 + \dots$$

(any analytic functional can be represented in such form). Axioms for  $S$  are as follows. (1) Many-component source. Assume

$$I = I_1 + I_2, \text{supp } I_1 \cap \text{supp } I_2 = \emptyset, \text{supp } I_1 = \text{supp } I_2$$

We demand that

$$S(I_1) < S(I_2) \Rightarrow S(I_1 + I_2) < S(I_1 + I_2)$$

(if one component is fixed, the image is simpler if the second is simpler). (2) Translational invariance. For any translation  $T$ :  $S(I_1) < S(I_2) \Rightarrow S(TI_1) < S(TI_2)$ . (3) Independence on choice of unit for flux  $I$ : if  $S(I_1) > S(I_2)$  and  $I_1, I_2$  are consistent with the same observations (that is

$$\int I_j(\bar{\sigma}) A_j(\bar{\sigma}) = C_j \text{ for } j = 1, 2$$

and some fixed  $C_j, A_j, A_0 = 1$ ), then  $S(\lambda I_1) > S(\lambda I_2)$ .

In looking for the simplest values of  $I$  an additive constant in  $S$  is inessential. Assuming  $S_1 = S_2$  if  $S_1 - S_2 = \text{const.}$ , then one can prove the following. If  $S$  satisfies

points (1)–(3), then either

$$S(I) \sim K \int I \ln I \, d\bar{\sigma},$$

or

$$S(I) \sim K \int I^\alpha \, d\bar{\sigma}$$

for some constants  $K, \alpha$  ((1) leads to  $S_2 = S_3 = 0$ , (2) leads to

$$S(I) \sim \int \tilde{S}_1(I(\bar{\sigma})) \, d\bar{\sigma}$$

for some  $\tilde{S}_1$ ).

MEM is usually chosen, because in all other cases  $I \geq 0$  is an extra complication and here it follows automatically from the variational principle.

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**FIDDY AND GREENAWAY REPLY**—We thank Kreinovič and Kosheleva for their remarks concerning our criticism<sup>1</sup> of phaseless object reconstruction using the maximum entropy algorithm<sup>2</sup> and other statistical techniques. The paper giving a more detailed explanation of our criticism is not widely available<sup>3</sup>. Because of this, we are taking the opportunity to supplement our necessarily brief discussion<sup>1</sup>, and to comment on the points which they have raised.

Their statement in the paragraph beginning “No matter how precise . . .” is an oversimplification. The claim may be true if the multiple sources in question are distributed over a region unresolved by the measurement baseline. However, if the measurements are precise, one may analytically continue the data and thus amend the resolution criteria. Also, the use of entire function theory enables one to predict a countable, complete and often finite set of possible solutions, from precise measurements. In ref. 3 we give examples of the possible solutions consistent with an intensity distribution. In only one case do all the solutions correspond to a double source. The essential point, which is apparent from the other examples, is that even within the given resolution criteria for the truncated data, there is the danger of confusing single and multiple sources when making phaseless reconstructions.

To say that one only requires “the distribution that contains . . . the minimum possible number of components”, implies lack of interest in multiple systems. If only upper limits on source extent are required, one may use the measured object autocorrelation. If the source structure is of interest, it is important to ask what the ambiguities are, and to classify them as

possible single, double, triple systems. Entire function theory predicts that such a classification is possible, the range of solutions being countable.

We do not deny the usefulness of the maximum entropy method, since with phase information it provides an extremely powerful tool. Gull and Daniell<sup>4</sup> note the possible existence of several entropy maxima and comment that in such cases the reconstruction “will be misleading”. They continue “with phase information . . . this cannot happen”. We concur, believing that such multiple entropy maxima correspond to ambiguities of the type discussed in refs 1 and 3.

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## Effects of sodium butyrate on human chronic myelogenous leukaemia cell line K562

ANDERSSON and his colleagues have postulated that the K562 cell line is of erythroleukaemic origin<sup>1</sup> and can be induced to undergo erythrocytic differentiation by sodium butyrate<sup>2</sup>. However, treatment with sodium butyrate of the original K562 cells fails to produce benzidine-positive staining, cell haemoglobin crystals or the synthesis of human globin.

The K562 cells (initial density  $2 \times 10^5$  cells  $\text{ml}^{-1}$ ) were cultivated in medium described elsewhere<sup>3–6</sup> in the presence or absence of 1 mM sodium butyrate. In contrast to Andersson's observation, sodium butyrate inhibited the cell growth by 50%. Thus, after 3 d, control cultures contained  $8.7 \times 10^5$  cells while butyrate-treated cultures had  $4.3 \times 10^5$  cells. No differentiation was detectable. The Lepehne reaction did not reveal a single benzidine-positive cell. We did, however, observe that cells from cultures treated with butyrate showed various signs of degeneration such as vacuolation, hyaline degeneration and loss of the cytoplasmic basophilia. Electron microscopy also showed various signs of cell injury.

In the normal sequence of erythroid differentiation the cell progresses through a series of definitive recognisable stages culminating in a mature erythrocyte.

Andersson *et al.*<sup>2</sup> showed the intracytoplasmic generation of erythrocyte-like bodies which were subsequently expelled from the original cell. This phenomenon has not been described before and these ‘erythrocyte-like’ particles are probably only hyaline bodies and/or pieces of cytoplasmic debris. Furthermore, neither treated nor control cells synthesised  $\alpha$ - or  $\beta$ -globin chains (Fig. 1) as would be expected if erythroid differentiation has occurred. With the report of Rutherford *et al.*<sup>7</sup> in mind, note that embryonic globin polypeptides are not detectable by the column chromatographic procedure used. Thus, it is not possible to exclude the possibility of synthesis of trace amounts of embryonic globin polypeptides, but it seems convincing that adult globin was not synthesised in cells treated with butyrate.

Because our K562 cells do not stain positively after exposure to sodium butyrate we also studied the effect of sodium butyrate in myelomas made up of K562 cells transplanted in nude mice<sup>8,9</sup>. Six intraperitoneal injections of sodium butyrate (275 mg per kg every 2 d) produced extensive necrosis of the myeloma. Imprints from K562 tumours growing *in vivo* in nude mice also failed to show benzidine-positive cells. However, when we attempted to reculture the cells from tumours, ~10–15% of the cells did give positive benzidine staining. These cells were not typical erythroid cells and showed well defined nucleoli. It is most likely that they were of mouse origin or the K562 cells were somehow altered by passage in the mouse to give a false positive benzidine reaction. If Andersson's cells have at any time been passaged in immunodeficient mice, this may account for the benzidine-positive cells. We do not know which clone or subline of K562 was used by Andersson *et al.*<sup>1</sup>. In March 1978, we sent to Dr Eva Klein a clone of K562 which was recultured *in vitro* after being serially transplanted in nude mice for 20 passages. Klein also had a subline of K562, originally provided to Dr G. Henle six years ago, and the myeloid origin of this subline was confirmed by Klein *et al.*<sup>10</sup>. Evidence for the myeloid nature of K562 cells is further supported by the presence of group-specific granulocyte antigens<sup>11</sup>.

Another claim of erythroid origin rests primarily on the biosynthesis of glycophorin A, the major sialoglycoprotein of human erythrocytes, by K562 cells<sup>12</sup>. Unpublished results show a very low number of glycophorin A cells in our K562 cell line and no conclusions can be made yet.

In summary, the failure of sodium butyrate to induce erythroid differentiation of K562 cells is consistent with our earlier studies on the effects of dimethyl sulphoxide<sup>13</sup>. Both agents produce cytotoxic effects rather than a stimulation of the cell potential for differentiation. The discrepancies between our findings lead us

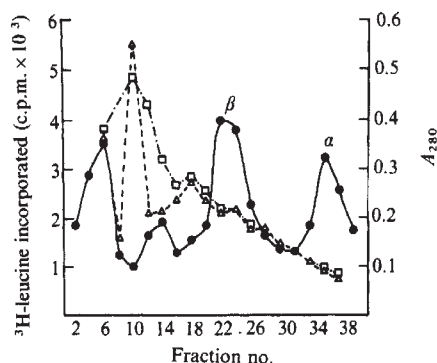


to question whether Andersson's cells are indeed K562 cells. The K562 cell line was originally established in our laboratory from a pleural effusion of a patient with chronic myelogenous leukaemia in terminal blastic crisis<sup>3-5</sup> and has been found to be a highly undifferentiated cell of the granulocytic series<sup>5,6,10,11</sup> with a consistent ultrastructural pattern<sup>14</sup>. It is the progenitor of all K562 sublines now in existence. The possibility of spontaneous changes through clonal evolution or of

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**Fig. 1** Elution profile of globin chains on CM-cellulose-urea columns. Absorbance of globin chains from carrier human haemoglobin (●) is shown in comparison to the incorporation of <sup>3</sup>H-leucine into polypeptides obtained from control cells (□) and from K562 cells incubated in the presence of 1 mM sodium butyrate (△). After addition of label at 48 h cells were pelleted at 12 h of incubation and then washed in excess saline<sup>15</sup>. H<sub>2</sub>O (1 ml) and carrier human haemoglobin was added. Globin was prepared by precipitation in acid acetone (1.2% HCl in acetone)<sup>16</sup>. The lyophilised globin was resolved into constituent polypeptides by chromatography on CM-cellulose columns in 8 M urea<sup>17</sup>. A phosphate gradient from 0.005 M to 0.03 M was used and 2.5-ml fractions collected. Aliquots (0.5 ml) were then used for scintillation counting.

contamination with other cell lines and subsequent overgrowth or hybridisation cannot be discounted after the cells have been grown outside our laboratory for so many years. Characteristics lacking in the parent cell line may be present in a subline which acquired them at some stage after leaving our laboratory. In any event, the classification of the K562 cell line as an erythrocytic precursor is certainly premature for the evidence available. The presence or absence of glycophorin A in highly undifferentiated precursors such as the stem and differentiating cells has not yet been established and so it should not be considered solely as a marker of the erythrocytic series. Morphologically, the K562 blasts are probably closer to haemocytoblasts than pronormoblasts or proerythroblasts depending on the terminology used.

ANDERSSON ET AL. REPLY—Lozzio *et al.* challenge our conclusion that the K562 cell line has the capacity for erythroid differentiation and raise doubts that we have studied the authentic K562 line.

The cell line was originally obtained from Lozzio via W. Henle. A detailed account of its characteristics was given by Klein *et al.*<sup>1</sup>. The karyotype of our K562 cells is obviously identical to that originally described by Lozzio and Lozzio<sup>2</sup>. Contamination with mouse cells or with mouse/human hybrids can be excluded, as the cells have never been carried *in vivo*.

Concerning the erythroid versus granulocytic nature of the K562 cells, we have demonstrated that: (1) The surface glycoprotein pattern of K562 cells resembles that of normal erythrocytes<sup>3</sup> but is completely different from those of other haematopoietic cell lines<sup>4,5</sup> of the promyelocytic leukaemia line HL-60<sup>6</sup> and of granulocytes<sup>7</sup> and their benign and malignant precursors<sup>8</sup>. (2) Our K562 cells carry on their surface  $1.1 \times 10^6$  glycophorin A molecules per cell<sup>9</sup> which are synthesised by the cells<sup>10</sup>. (Glycophorin A is the major sialoglycoprotein of human red cell membrane and is present only on erythroid cells in human bone marrow<sup>11</sup>.) (3) K562 cells synthesise haemoglobin in the presence of sodium butyrate<sup>12</sup> as shown by the Lephene's benzidine and by radioimmunoassay indicating the presence of both the haem and the globin parts. Rutherford *et al.* recently reported

that K562 cells synthesise embryonic and fetal haemoglobin in the presence of haemin<sup>13</sup>. The identities of the haemoglobins have recently been confirmed by peptide mapping (T. R. Rutherford, personal communication).

The different observations on phenotype of the K562 cells by us<sup>12</sup> and Rutherford *et al.*<sup>13</sup>, as compared to those by Lozzio and Lozzio<sup>2</sup>, are not easily explained. As the original K562 cell line was not initially cloned the possibility exists that we have been investigating selected subpopulations of the cell line endowed with different differentiation capacities. (This is also frequently seen in the Friend leukaemia system.) Therefore we suggest an interchange of our respective K562 cells for comparative studies.

Regardless of the real phenotype of the K562 cells, the establishment of this unique cell line by Lozzio *et al.* is highly creditable. This has provided an excellent model for human haematopoietic differentiation.

**Note added in proof:** Recently we obtained K562 cells (passage 239) directly from Lozzio's laboratory (courtesy of Dr R. A. Laine). These K562 cells have a strong surface expression of glycophorin A as shown by immunofluorescence and immunoprecipitation from surface labelled cells with specific antiglycophorin A antiserum.

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# reviews

## Management of controversy

Eric Ashby

*Controversy: Politics of Technical Decisions.* Edited by Dorothy Nelkin. Pp. 256. (Sage Publications: Beverly Hills and London, 1979.) Hardback \$16, £10; paperback \$7.95, £5.

SCIENCE-AND-SOCIETY, hyphenated like this, is now a well established subject for study, with departments, professors, journals, conferences, research grants: all the apparatus of an academic discipline except a codified content. This is not a pejorative assertion. A codified content takes time to evolve; premature generalisations might become obstacles to evolution; the most helpful contributions to make at this stage are in the 'natural history' of the subject: descriptions of what actually happens (the echo of Ranke's recipe for writing history "*wie es eigentlich gewesen*") and cautious comparisons of what seem to be similar episodes.

This is what Dorothy Nelkin has been doing for some time. She has published case-studies of controversies about the siting of a nuclear power station, about the extension of the Boston airport, about housing politics, about military research at MIT; all controversies: for the first lesson the novice has to learn in this subject is that sweetness-and-light is not only impracticable, it is undesirable. Controversy is essential to the resolution of the problems that arise from the applications of technology to social ends. It's a consequence of political freedom. So attention has to be given, not to the elimination of controversy but to its management. Who has the right to take part? How can those who take part have access to information? What is the appropriate forum for the controversy: a court of law, a ministerial public enquiry, a panel of experts, a clash of pressure groups? Experiments which might provide answers to these questions are going on all the time. The Royal Commission on Electric Power Planning in Ontario has published some notable material; so has the Electric Power Research Institute in California; so, in Britain, has the Advisory Committee on Trunk Road Assessment. But the most useful material for the student is a collection of documented case-studies, as little coloured by prejudice as is possible in a subject where exaggeration, sensationalism, and distortion of the facts are the very raw material in the process.

This is what Dorothy Nelkin has achieved in her editorship of this useful

collection of twelve case-studies in the politics of technical decisions. The studies are intended to "provide students with a sense of the kind of reasoning that motivates public agencies, government officials, scientists, and protest groups". She distinguishes four kinds of controversy and the contributors to the volume give three examples of each kind. They are: efficiency versus equity, as in the siting of an airport; benefits versus risks, as in the controversy over nuclear wastes; regulation versus freedom of choice, as in the prescription of certain drugs not because they are dangerous but because they appear to be useless; and science (the rational approach to nature) versus traditional values (opposition to the pursuit of knowledge irrespective of where it might lead.) Each case-study is well supported by references to primary sources.

I would like someone to write a monograph on controversies about airports. Heathrow is a shameful example of the incompetence of the British in the planning of air transport and the futility of the present state-of-the-art of technology assessment. But we have no monopoly of incompetence. In the 1950s there was a vast investment in Gander airport in Newfoundland, "just before the introduction of jet aircraft made nonstop trans-Atlantic flights possible". Then, as a case-study of the Toronto airport goes on to explain, came the proposal to site a second airport in Toronto. One motive was rivalry with Montreal; if Toronto didn't expand, Montreal would become the "gateway to Canada". Another motive was to pacify people living near the present airport. Opposition to a new airport developed rapidly, just as it is now doing in Britain to oppose any site for a third London airport. It coalesced under a good emotive label: POP — People or Planes. Among the supporters of the opposition was one aircraft manufacturer, who saw a better market for short take-off craft if there were to be only one airport in Toronto. One interesting and significant point which the essay makes is that the real issues were political but they were often camouflaged as scientific or technical.

The most interesting essay in the book, because it draws upon almost inaccessible sources, is one on environmental conflict in the USSR. With a wealth of references, almost all Russian, Thane Gustafson describes the awakening of an

environmental conscience in the Soviet Union. He effectively dispels any notion that it came from the grassroots. But he shows that the genuine concern about the condition of Lake Baikal was not solely for commercial expediency: "Through literature and folklore, every Russian from childhood knows its beauty". And this was a powerful factor in the campaign to clean the lake. Concern for water management and opposition to the siting of dams arose because it was causing a serious alienation of valuable agricultural land. But the new environmental programme owes its impetus to the influence of scientists, aware of the concern for conservation in Europe and America, who have made themselves audible in the councils of the Kremlin.

All the case-studies are interesting and a few comments on them will illustrate how well they have been chosen. The familiar ban on the use of diethylstilbestrol (DES) is an example of the need to balance the benefits of more meat against the potential disbenefits of cancer: one a certainty, the other an unproven possibility. It is noteworthy that the Food and Drug Administration avoided justifying the ban on grounds of the notorious Delaney Clause because President Nixon proposed to Congress that this clause should be "diluted" to avoid some of the more crazy consequences of its application "to ban all foods because they contain a few molecules of naturally occurring carcinogens".

There is a historical chapter, showing that controversies about the uses of science are nothing new, which traces the opposition to smallpox immunisation in the eighteenth and nineteenth centuries. And there is an ominous account of an attempt — yes, as late as the 1970s — to give equal treatment to the theories of evolution and of special creation in the teaching of biology in California. California buys about 10% of the nation's textbooks and it was seriously proposed that special creationists should have at least equal consideration in textbooks with the consideration given to orthodox biology. The National Academy and the American Association for the Advancement of Science had to throw their weight into the controversy. Perhaps we should not be surprised; astrologists can still make a good living in Britain and it is often said that more people make a living out of astrology than out of astronomy.

This book is a useful addition to a small,



but growing, literature of case-studies of this kind. By far the best, though its scope is limited to planning enquiries, is Roy Gregory's *The Price of Amenity* (Macmillan, 1971). There is also, for British examples, the book edited by Peter Smith, *The Politics of Physical Resources* (Penguin, 1975), though some of the essays in this book are far from objective; and a book edited by R. Kimber and J.P. Richardson, *Campaigning for the Environment* (Routledge and Kegan Paul, 1974). The most comprehensive summary, though the case-studies are unfortunately

very brief, is in the book edited by Edward Lawless, published by Rutgers University Press in 1977 (reviewed in *Nature*, 274, 295-296; 1978) under the unpromising title *Technology and Social Shock*.

So there is now the nucleus of a library which will promote the evolution of science-and-society into a respectable discipline. Dorothy Nelkin's latest contribution to this is very welcome. □

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## Encyclopaedic approach to technology

*A History of Technology*. Vols VI and VII: *The Twentieth Century, c.1900 to c.1950*. Edited by T.I. Williams. (Clarendon/Oxford University Press: Oxford, UK, 1979.) £25 and £27.

TWENTY-FIVE years ago the now classic first volume of *A History of Technology* appeared from the Clarendon Press. This year, we welcome the sixth and seventh (and last) volumes in the series. With them we arrive, in history, where the series itself began, in the early 1950s. Together, these seven magnificent volumes form a monument of scholarship. Monuments normally inspire criticism or veneration; rarely use. This one is different.

From the early days of his collaboration with Charles Singer, E.J. Holmyard and A.R. Hall, Williams enjoyed the cooperation of a legion of eminent scholars whose unquestioned technical expertise made their contributions definitive. In editorial standards, range and clarity, the present volumes continue their predecessors' tradition of high scholarship. Nonetheless, 25 years is a long time in historiography, as in technology. Moreover, a new generation (and profession) of historians has emerged, with significantly altered perceptions of the social function of technology and technologists. With them, the reader is compelled to ask in these more cautious times, has the history of technology progressed as far as the technology it describes?

In reply, these two volumes invite comment at three levels. First, these chapters are, in technical terms, proverbially encyclopaedic in breadth and detail. Like their predecessors, these volumes describe a spectrum of activity, from fuels to food, metals to textiles, chemicals to buildings and transportation. Some chapters bring the story 'up to date'; others (space, aircraft, medical and ocean technology, and computers) describe recent additions to the "relief of man's

estate". Although experts may differ (a possibility frequently raised, if indifferently addressed), one can scarcely debate the pronouncements of Lord Montagu of Beaulieu on motor cars, or of Colin Buchanan on town planning. Several other chapters — including Frank Greenaway's on instruments and Brian Bowers' on electricity — offer fresh insights as well as information. But these technical chapters together seem less than the sum of their parts. There is, despite the editor's catholicity, a deceptive provincialism of outlook which emerges in many of them. A mixture of "practical men" and university men (in about equal parts) is not necessarily an unsatisfactory formula for a fine product; but the fact that the contributors are overwhelmingly British, and, with perhaps ten exceptions, are not professional economic or social historians, points clearly to one difficulty. Another lies in the subject-matter itself.

One cannot criticise the authors for failing to make obligatory reference to microelectronics or biotechnology (although, indeed, there are none). But it will not escape notice that many of the 58 contributions (notably excepting the editor's own) follow historiographical conventions remarkably similar to those of educated amateurs a generation ago. Most technical chapters are history preserved in aspic, sadly neglecting systematic comparative analytical discussion. Excellent descriptive accounts of leading technical developments in different fields may nonetheless strike many a current reader, of say, "Technology and Culture", as dry, stylised and lacking in economic and social insight. On the one hand, few of the technical chapters even attempt to demonstrate the connection between entrepreneurial and management styles in research and development, and the problems of marketing and diffusion in different countries under different economic systems. On the other hand, few contributors pause to ask, how can we use this knowledge to understand the developments of the *next* twenty years?

Second, as the editor explains, technology advances across a broad front, or not at all. Yet the editorial arrangement of technical contributions, inevitably dividing knowledge into arbitrary

categories, militates against the authors asking larger questions about the nature of the technologies concerned. Today, whatever may be one's opinion, for example, of 'appropriate technology', the reader will want to know in what ways technological development (whether in fuel, transport, housing, and so on), has been shown capable of responding to altered economic and social realities. It could be argued, in defence, that in the 1950s, most scholars and the English reading public were as uninterested in the relationships of production as they were unaware of the implicit risks of technological development. But whatever one's approach to economic history, no account of technological development which omits to describe the organisation and operation of the modes of production characteristic of industrial capitalism can today be considered complete.

If this criticism is sound, it is even more to be regretted, as the editor is clearly sensitive to the issues involved. Indeed, for this reason, Mr Williams has gone outside the ranks of the technological *cognoscenti*, and has invited several chapters of contextual discussion — including an historical overview from T.K. Derry, and chapters on innovation by David Sawers; on the economics of technology by Frank Bradbury; on management by Glenn Porter; on trades unions by Harold Pollins; on education by David Layton; on government by Alexander King; and on the chemical industry by Lutz Haber. These contributions are of varying quality (Haber's and Layton's in particular deserve a wider readership), but whatever their individual merits, they do not together weave a comprehensive fabric. Written, one supposes, in isolation from each other, they largely fail to reveal the depths of controversy reigning within these areas of discourse. The explanations offered are, in many ways, too consensual, too safe, and altogether too undemanding. When they jar, then, like a clock striking thirteen, they cast doubt on all else. For example, David Sawers concludes provokingly that "the nature of . . . technology appears to influence the nature of the industrial structure more than the structure influences the technology". Surely, the two volumes of evidence which follow could, if skilfully used, demonstrate, disprove, or at least illuminate this assertion. At the very least, the economic and social analysis of new technologies cannot be merely relegated to separate chapters. At a more philosophical level, far more could be said about the relationship between technology and cultural expectations. For example, it could be argued (with important qualifications) that a presumed alliance between liberal values and technological growth was widely considered axiomatic in the 19th century; but Derry's passing reference to this fails to demonstrate why this should have been so, or what relevance

this belief in "technologism" had to subsequent political and social development. Here and elsewhere historical judgements will not escape historical criticism. And in the meantime, many important relationships — between technology and empire, between political and "technological dependence", between civilian and military technology, and between the "technostructures" of different economic systems — are simply not adequately treated.

Browsing through these excellently produced volumes, the reader may, at length, ask a third question. Social history, Trevelyan once remarked, is history with politics left out. The history of technology is, traditionally, history with the people left out. Beyond inventors, corporations and eponymous effects, this *History of Technology*, like its predecessors, largely fails to situate technological developments at the level of human beings caught up and affected by them. This separation between the word and the thing has until recently rendered much of the history of technology an arid discipline, often bereft of human reality. It has been by all accounts a *hasard du métier*. But the time has come to catch up, at least, with recent historiography. In these volumes, revealingly, the excellent photographs wordlessly describe much of the reality that the text leaves unstated. Even to the casual reader, pictures of working conditions in the paint industry (Fig. 23.3) rest uneasily next to discussions of chemical development. And one supposes that the plate (Figs. 21.15 and 21.16) which offers an ironic (and unintended?) juxtaposition of *men* in lab coats in Levenstein's Manchester works (about 1912) alongside *women* in overalls cleaning chemical casks (about 1943) will not pass unnoticed by students of technology and the labour process. Nor, for that matter, will feminist historians leave unchallenged the chapter on domestic appliances, which manages to describe a host of new inventions, and mentions more than once the "servant class", without once referring to the commonplace contradictions of "liberating technology" in modern society.

Methodologically, it can be argued that the omnibus, encyclopaedic approach to technology represented in these volumes, has had its day. What of the future? In the last chapter, written by the editor, there is a beginning. Mr Williams, as a good historian, looks forward, and in his summary he sees the chief lesson of the past and the problem of the future as basically one of choice — one of selection — between different technologies. Other historians, economists and even technologists today would argue that the real problem, no less pressing in the Third World than in the West, is not merely one of choice between different technologies, but of choice between different styles of life. Mr Williams sees clearly the difficulty of first defining an acceptable goal, then

creating the technology necessary to achieve it. But to do something like this, after all, is surely part of the technologist's credo. And in failing to set and observe clear social priorities, technology in the West has been allowed to become less a means and more an end in itself.

Historians now widely recognise that it is the fate of technology not to have an ideology, but to be one. As Langdon Winner has recently observed, the fear that technologies and the factors governing them have become 'autonomous' outside the rational control of governments, or engineers, is part of the Western condition. There is no reason, on current calculations, why this fear should not also one day confound large parts of the Third World. Since *Silent Spring*, the environment, and since the oil crisis, energy, have become the two principal epicentres of disquiet with technology. But the historical and philosophical issues raised in *A History of Technology* are not confined to these matters alone. That these last volumes are edited by Trevor Williams alone, no longer

aided by a large industrial subsidy, itself speaks volumes for the change in our circumstances.

When they turn back to his *History* for technical answers, readers of these volumes will be left asking important questions. One hopes they will find the editor sympathetic. Is it sufficient, today, for example, to deplore alleged 'constraints' on 'progress', when one is forcibly reminded almost every day of the traumatic story of nuclear power from Los Alamos to Harrisburg? If not in the 1950s, then from the 1970s some conception of 'open' technology will require a place alongside participatory access to 'open government'. In the process it is not only the technologist and the politician, as Mr Williams suggests in an adventitious pun, whose resources will be taxed in the search for solutions. If the future lies in the discovery of alternatives, solutions can lie only in ourselves.

**R. M. MacLeod**

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## Hot-atom reactions

*Chemical Effects of Nuclear Transformation in Inorganic Systems.* Edited by G. Harbottle and A. G. Maddock. Pp. 540. (North-Holland: Amsterdam, London and New York, 1979.) \$87.75; Dfl. 180.

ATOMS undergoing almost any nuclear process are characterised by excitation energies usually far in excess of the energies associated with chemical processes. The excitation may be in the form of kinetic or electronic energy (including ionisation), the amounts depending on the nature of the nuclear process occurring. If the process gives a radioactive atom, the atom is conveniently 'tagged' at birth and its subsequent fate readily followed.

The chemical effects of the nuclear process are firstly to cause rupture of some or all of the bonds in which the atom was involved and secondly to allow the atom to undergo novel types of reaction not normally observable in conventional chemical systems. These are normally called 'hot atom' reactions, as the excitations involved correspond to 'temperature' of the order of  $10^5\text{K}$  or higher.

The present volume is a comprehensive survey of these reactions occurring in inorganic systems, up to the mid-1970s. It has 27 chapters contributed by 28 authors and lists some 1900 references in total. The limitation to inorganic systems may seem somewhat arbitrary, but in fact the coverage of the whole field is good and few if any topics of fundamental importance are omitted.

After a short historical introduction the first few chapters deal with the primary processes following nuclear activation and reactions in the gas phase. Gas phase studies have contributed significantly to our understanding of high energy reactions through the work of Wolfgang, Rowland and others. However, as most inorganic compounds are not gaseous, the vast majority of the work covered here deals with the condensed phases, especially solids. Much of the work on solids has been stimulated by the phenomenon of 'annealing' in which the activated atom, having undergone bond rupture during nuclear activation, returns to the form of the parent compound as a result of a variety of treatments, such as heating or gamma irradiation.

The division of the chapters is made on broadly chemical criteria, so that each group of compounds is treated separately. This makes sense because by far the most popular method of nuclear activation is the  $(n, \gamma)$  reaction and there are separate chapters dealing with other nuclear processes such as isomeric transition, beta decay, fission fragments, and so on.

A major experimental problem in studies on solids is the need to dissolve the solid in order to analyse products. The possibility of reactions during or after dissolution makes it difficult to be sure of the precise nature of the species present in the solid. Numerous attempts to overcome this difficulty have been made and some of these are reported here. One obvious approach is direct studies on the solid itself, but this is difficult because of the exceedingly low concentration of radioactive atoms usually present. Mössbauer and related techniques have yielded some information in this area, but



by no means solve the problem.

In summary, this volume provides a comprehensive study of the state of the art in this subject. It accurately reflects the uncertainties as well as the progress made. The standard of treatment is somewhat variable, as might be expected with such a large number of contributors, but the

editors have done an excellent job in avoiding overlaps and repetition. The price will probably put the book beyond the reach of even the most enthusiastic individual, but it should certainly find its place in libraries.

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## Energetic Mancunian

*James Prescott Joule and the Concept of Energy.* By H.J. Steffens. Pp. 172. (Neale Watson: New York; Dawson: Folkstone, UK, 1979.) \$20; £10.

NEARLY a century ago a reviewer in the *Philosophical Magazine* wrote:

"Not Copernicus and Galilei, when they abolished the Ptolemaic system; not Newton, when he annihilated the Cartesian vortices; not Young and Fresnel, when they exploded the Corpuscular Theory; not Faraday and Maxwell, in their splendid victory over *Actio in distans* — more thoroughly shattered a malignant and dangerous heresy, than did Joule when he overthrew the baleful giant FORCE, and firmly established, by lawful means, the beneficent rule of the rightful monarch, ENERGY! Then, and not till then, were the marvellous achievements of Sadi Carnot rendered fully available; and Science silently underwent a revolution more swift and more tremendous than ever befell a nation".

None of this Victorian enthusiasm for the Manchester brewer's son is conveyed by Henry Steffens, nor does he attempt to explain the significance of the change from "force" to "energy" in physics. He has compiled instead a sober survey of Joule's experiments and theories from 1837 to 1852, together with a discussion of closely related contributions of J.R. Mayer, William Thomson, W.J.M. Rankine, R. Clausius and H. von Helmholtz. Steffens elucidates the path from early experiments on the "electromagnetic engine" to determinations of the mechanical equivalent of heat and formulations of the first law of thermodynamics. Although it provides a clear and accurate account of one aspect of the history of energy conservation, the book suffers from its limited scope. We learn little about Joule's personality or his influence beyond the small circle of scientists mentioned above, and scarcely anything about the concept of energy apart from Joule's rather narrow interpretation of it.

Steffens' most interesting chapter is the one dealing with Joule's rough translation, in his notebook, of Mayer's 1842 paper; he argues that Joule knew of Mayer's work before 1844 and was thereby led to perform the experiments on rarefaction and condensation of air that yielded his third value for the mechanical equivalent of heat. But aside from this, Steffens makes very little use of documents, even those already published (for example, by A.L. Hatton and L. Rosenfeld in 1956, and by J.T. Lloyd in 1970).

This book is almost identical to the author's 1968 dissertation, with the addition of four pages on the paddle-wheel experiment and the deletion of some material on Mary Somerville and W.R. Grove in chapter 6. Much has been written on nineteenth-century physical science during the past decade and as a result historians of science have not only a different view of the development of energy concepts but an enhanced interest in philosophical and sociological questions. Steffens mentions some of the relevant publications in his notes but has made few substantive changes in his text. Perhaps most serious is his failure to take account

of John Forrester's important article on the chemical background of Joule's work (*Studies in History and Philosophy of Science*, 1975). Ten of the 22 items in Forrester's "Joule bibliography" are missing from Steffens' bibliography (users of the latter should be warned of a mixup of lines of type on pages 165-66).

It is necessary to criticise two features which this book shares with an increasing number of scholarly monographs. First, if notes must (because of the deterioration of printing technology) be placed at the end of the book instead of the foot of the page, they should be keyed to the text pages so one does not have to find out what chapter one is reading in order to locate its notes. Second, the index should cover the notes as well as the text; in this case it doesn't even cover the text adequately (for example, Aepinus has no entry).

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## Liquid crystalline materials

*Liquid Crystals and Biological Structures.* By G.H. Brown and J.J. Wolken. Pp. 187. (Academic: New York, San Francisco and London, 1979.) \$19; £12.35.

It is surprising that many distinguished scientists, including Bernal in 1933 and Needham in 1950, should have pointed out so early the importance of the liquid crystalline state for biological systems. Yet it was only in the early 1960s that a major attempt was made to examine experimentally the liquid crystalline properties of biological molecules. Nowadays when so many scientists have contributed to the study of phospholipid-water systems to provide insight into biomembrane structure and function, it is interesting to remember that a great deal of the basic fundamental work — for example, phase transitions, phase diagrams, fluidity, bile salts and cholesterol effects — was carried out as recently as the 1960s. When we further note that myelin figures were first observed by Virchow in 1854 it is interesting to consider the socio-economic technological factors which held up the study for so long of these important liquid crystalline lipid-water systems.

Brown and Wolken remind us of this in the present book. They examine the basic properties of liquid crystalline materials and include a discussion of the various classifications, including nematic, cholesteric and smectic liquid crystals. Thermotropic and lyotropic liquid crystal and the neat soap phase important to the

detergent industry is shown. The optical properties of liquid crystals are also adequately but briefly described. These chapters I found neat, and useful. However, the later chapters dealing with the relationship of these fundamental properties of matter to the biological structures are less satisfactory.

The authors include chapters on the cell, photoreceptor structures (that is, chloroplasts and the eye, fibrous protein structures and effectors, and biomembranes). They also include applications to medicine, such as temperature scanning and diseases. I found these biological chapters a little too superficial for my taste. I would have preferred some better examples of how important an understanding of liquid crystals has been for their biological implications — for example, the way in which thermotropic phase transitions which occur in pure phospholipid-water systems are clearly observed in some natural biomembranes. The use of liposomes as potential pharmacological capsules and the formation of gall-stones are also given only passing reference, and yet these are interesting, useful and important applications of the study of liquid crystalline substances to biology and medicine.

As an overview providing a stimulus for a student or young research worker the book may be valuable. It is clearly written with good diagrams and printing. I observed only a few obvious typographical errors.

Dennis Chapman

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# obituary

## Sir Ernst Chain, 1906-1979

SIR ERNST BORIS CHAIN, who received the Nobel Prize for Physiology and Medicine with Fleming and Florey in 1945, for the discovery and isolation of penicillin, died on 12 August 1979 while on holiday at his country home in Ireland.

He was born in Berlin of a Russian emigré father and a German mother. On 30 January 1933, Chain left his native Berlin to escape from the mounting evils of Nazi Germany. He arrived in London with little but hope and a determination to follow one or other of his great passions — music or science. After a short interlude at University College London, Chain had the good fortune to be recommended by J.B.S. Haldane to Sir Frederick Gowland Hopkins, then Head of the Sir William Dunn School of Biochemistry at Cambridge. Chain was overjoyed with this unexpected opportunity which he seized eagerly: the die was cast on the side of science rather than music.

The topic which engaged Chain's interest was the biochemical basis of the neurotoxic effects of snake venoms. At Cambridge, Chain found that some of the most potent venoms were able to inhibit alcoholic fermentation and glycolysis when added in very small amounts to cell-free extracts. Further studies showed that this was due to destruction of an essential coenzyme by simple hydrolysis. This approach of biological observation followed by biochemical explanation was symptomatic of Chain's scientific philosophy which he followed consistently through 45 years of amazingly diverse achievements.

After two years of working with 'Hoppy', Chain was approached by Professor Howard Florey with an invitation to join Florey's staff at the Sir William Dunn School of Pathology at Oxford. Florey had no biochemical training but he did have an awareness of the importance of biochemistry in the understanding of all biological sciences and in particular of his own field of experimental pathology. Florey drew Chain's attention to an observation by Fleming in 1924 concerning the lytic properties of tears, nasal secretions, and egg white on bacteria. Such a problem — to study the biochemical mode of action of a powerful bacteriolytic agent — was typical of the Chain approach. He was able to show that the lytic properties were due to lysozyme which acted upon a bacterial



*Chain in Oxford, c. 1940*

polysaccharide.

While preparing this work for publication in 1937, and arising from discussions with Florey, Chain stumbled upon the concept of growth inhibition by bacteria, fungi and yeasts acting on one another, including Fleming's neglected original report on the bacteria-inhibiting properties of *Penicillium notatum*. Upon starting their systematic investigation of the antibacterial substances produced by micro-organisms, Florey and Chain were immediately embarrassed by a severe shortage of funds compounded by the refusal of the Medical Research Council to fund the work. One day in the autumn of 1937, Chain received a message from Florey stating that in view of the fact that the department had an overdraft of £500 no further equipment of any kind was to be ordered — not even a glass rod!

This experience had a profound effect on the young, enthusiastic Chain who was impatiently waiting to unleash his energies on the new problem of explaining in chemical terms how bacterial growth was inhibited. The unsuccessful approach to the Medical Research Council reinforced his suspicions about university and government financial support. Since that

day, he never allowed himself to become wholly dependent for research funds on political largesse, however respectable its facade. Undeterred by this initial setback, Chain discussed with Florey a grant application to the Rockefeller Foundation to cover the costs of the proposed programme for a number of years, so that financial stability could be ensured. To Chain's great delight, the Rockefeller Foundation were sympathetic towards biochemical research and granted him \$5,000 for five years.

The penicillin story is well known as it culminated in a Nobel Prize for Chain, Fleming and Florey in 1945. Chain was not the type to rest on his laurels. He was incensed by the refusal of the MRC and Oxford University to consider any form of patent protection for penicillin which was subsequently developed commercially in America. Chain also became increasingly frustrated by the problems of obtaining adequate facilities for the fermentation of micro-organisms on a semi-industrial scale. The methods used initially to provide impure penicillin for laboratory studies and even clinical trials were crude. Chain was very outspoken about the amateurish approach in Britain which he contrasted



with the facilities available in the USA. He campaigned vigorously at all levels, for fermentation pilot-plant facilities, but he was met by the implacable inertia of the Establishment who made it very clear that such facilities were a matter for industry and had no place in an academic ivory tower. Chain poured scorn on the view that the size of the reaction vessels should determine whether the biochemical problem to be investigated was 'pure' or 'applied'. In the true British spirit of compromise, Chain was finally offered a single fermenter of moderate dimensions. Chain's reply was to ask if an organic chemist would be prepared to limit himself to the use of one Erlenmeyer flask!

More in sorrow than in anger, Chain accepted in 1948 an invitation to organise a department of biochemistry at the Italian State Institute of Health in Rome. The temptation was made irresistible by the promise that in Rome, an International Centre for Chemical Microbiology containing the controversial fermentation pilot plant with extraction and purification facilities would be made available. The magnificent facilities and the collaborations with Ballio, Dentice, Falini, Marotta (the Director of the Institute), Pocchiari and his colleagues, produced a rich harvest of publications in the new science of chemical microbiology and on the biochemical mechanisms of hormonal control. A constant stream of scientific visitors passed through Chain's laboratories in Rome, so that at any one time at least half-a-dozen different nationalities were represented.

At the start of his Italian era Chain married Anne Beloff in 1948. His wife, who had trained under Sir Rudolf Peters and Baird Hastings, had an active team of collaborators in Rome investigating the mode of action of insulin. During this period the Chains were delighted by the birth of their three children — Benjamin, the eldest son, and then twins, Judith and Daniel.

In 1940 Abraham and Chain had identified the enzyme penicillinase, which inactivates penicillin and which is produced, with great foresight, by numerous bacteria. Chain felt that the affinity of penicillin for this bacterial enzyme could be altered if the penicillin molecule could be chemically modified. Enormous effort had already been expended in the pharmaceutical industry in the search for chemical precursors acceptable to the *Penicillium* mould in the hope that novel penicillins with useful properties would be biosynthesised. Of these, phenoxymethylpenicillin (penicillin V) had turned out to be acid stable and useful clinically because of its absorption after oral administration. Apart from this limited success, some disillusionment had set in regarding the prospects of obtaining novel penicillins, and many laboratories had turned their attentions to more promising areas of antibiotic research.

Chain's idea was to improve upon nature by persuading the mould to grow *p*-aminobenzyl-penicillin and then using the chemically reactive amino group for further man-made chemical modifications.

Chain was approached in 1954 by the then Chairman of the Beecham Group, Henry Lazell, on the advice of the late Sir Charles Dodds, with a view to advising Beechams on entering the antibiotics field. Chain's enthusiasm and drive so impressed Lazell that Beechams soon found themselves with a fermentation pilot plant looking remarkably like the one in Rome, and a research programme aimed at modifying *p*-aminobenzyl-penicillin. A small team of scientists was sent to Rome by Beechams while the fermentation plant was being erected. During their stay in Rome in 1956, an interesting difference in the results of two assays of the crude microbial culture fluids was noticed. The chemical assay based on the behaviour of the fused thiazolidine- $\beta$ -lactam ring consistently gave higher results than the bioassay which required the full penicillin molecule for biological activity. An explanation of this finding followed when the team returned to Beecham Research Laboratories in 1957 when they demonstrated that the discrepancy was due to the presence of the penicillin nucleus (6-aminopenicillanic acid) which was subsequently isolated and characterised, (*Nature*, 183, 257, 1959). The gateway was now open to an enormous variety of chemically modified derivatives beyond all the original expectations based on the limited synthetic variations possible with *p*-aminobenzyl-penicillin.

The focal point of antibiotic research had moved back to England, and this coincided with a worsening of the political situation in Rome. In the course of a visit to Rome in 1958, Professor P.M.S. Blackett discussed with Chain the possibility of setting up at Imperial College, London, a comprehensively equipped biochemistry department modelled along the lines of the facilities available at the Institute. A further visit by the then Rector, Sir Patrick Linstead, led to a tentative proposal that Chain should return to Enland to re-establish biochemistry at Imperial College where the chair had been vacant since Chibnall left for Cambridge in 1943.

The cost of all the fermentation equipment, instrumentation, electrical and mechanical workshops, required to support and service the complex chemical microbiological research and metabolic biochemical investigations to be undertaken by Chain, was formidable and unheard of in the academic world outside the realms of nuclear physics. The scheme was shelved. However, Chain accepted the challenge of an apparently insoluble difficulty and campaigned vigorously to find independent funds for this imaginative project which complemented the already very strong chemical and chemical engineering interests of Imperial

College. As a result of Chain's efforts and Linstead's encouragement, the Wolfson Foundation provided £350,000 to establish the new Wolfson Laboratories, the Science Research Council granted £110,000 in support of fermentation research, and the Fleming Memorial Fund granted Chain £50,000 for basic medical research. Chain had not forgotten the lesson of the forbidden glass rod at Oxford!

Chain returned to take up his new appointment in October 1964 and the new laboratories were completed in the summer of 1965. The Medical Research Council had buried the hatchet by establishing a Metabolic Reactions Research Unit under Chain. Dr Anne Beloff-Chain also continued her studies on insulin and the control of carbohydrate metabolism in the new department as Reader in Metabolic Biochemistry. The financial independence which Chain secured from the very conception of the new department did nothing to endear him to the administrative establishment which found him unimpressed by exhortations to economise in lean years. Minor officials in the Science Research Council and University Grants Committee secretariat found their cautious criticisms of Chain's eminently unreasonable demands quoted, or even possibly misquoted, by Chain in direct conversations with the Treasury or the Prime Minister (according to availability). It would be wrong however to give the impression that Chain was a mere irascible crusader against bureaucracy. He had an affectionate regard for his circle of friends with whom he loved to share musical evenings in which their active participation was expected. He was devoted to his family and all visitors, however distinguished, were put aside, albeit temporarily, whilst Chain greeted his sons and daughter when they called in to his office on their way home from school.

The premature death of Sir Patrick Linstead was a sad personal loss to Chain and a watershed in the fortunes of the department. The sympathetic understanding which Chain had enjoyed with Linstead was irreplaceable and the special relationship of the department, because of its high level of collaboration with industry and support by charitable institutions, became almost an embarrassment in inter-college negotiations for funds.

Chain's knighthood came as something of a surprise in 1969 — not, of course, because of any doubts about his quite exceptional personal achievements over 35 years, but because his deliberate policy of rocking Establishment boats had made a deep impression in what are termed 'influential quarters'. It says something for the British political system that his scientific genius finally triumphed over his political reputation, even though 20 years earlier his election to Fellowship of the Royal Society had been delayed for several years.

After his retirement from the chair at Imperial College in 1973, he became Emeritus Professor. He continued to travel widely, aided by his ability to lecture in five languages. Honours and decorations came from many Universities and Governments including France, Italy and Japan, and Chain continued his long, active association with the Weizman Institute as a Governor.

An abiding memory for his family and friends must be the marvellous occasion in 1976 when Sir Ernst was guest of honour at a dinner held at the Middle Temple Hall to mark his 70th birthday. A crowded scientific programme had been given at the Royal Society over the preceding two days by his closest collaborators. This programme reflected Sir Ernst's research contributions in food technology and non-specific immunity as well as microbial and physiological biochemistry. Fortuitously news came through of his eldest son's first class honours in zoology at Cambridge. After dinner and the speeches (Sir Ernst almost lost for words for once), the entertainment for the distinguished international audience was opened by Sir Ernst and Benjamin Chain playing piano duets with great panache and empathy — a fitting reminder that the gain of science and medicine from the work and life of Ernst Chain was a loss to the concert hall and the world of music.

K. R. L. Mansford

## Sir Harry Champion

SIR HARRY CHAMPION, CIE, Emeritus Professor of Forestry in the University of Oxford, died on 19 June 1979. He was born in 1891 and was educated at the Royal Grammar School, Guildford. He was at Kings College, London University, for a year before going up, as a Commoner, to New College, Oxford in 1909. The subsequent award of a college scholarship was the first of his many academic achievements at Oxford. In 1912 he took a first in chemistry, in 1913 a first in botany and in 1914, under William Schlich, a Diploma in Forestry with distinction.

In 1912 he was accepted as a probationer in the Indian Forest Service and after spending a year in the United States on a Carnegie Travelling Scholarship to study entomology he went to India in October 1915.

Champion was appointed Assistant Conservator of Forests in the United Provinces, where P.H. Clutterbuck, later Inspector General of Forests, was Chief Conservator. At an early stage in his career he demonstrated clearly his versatility. In 1916 he was Acting Forest Entomologist at the Forest Research Institute, Dehra Dun, and in 1921 Silviculturist for the United Provinces. As a District Officer he compiled a check list of the fauna of the West Almora Division and also effectively modified the condensing equipment on

plant used to distill tar from Chir pine. For the latter he was commended by the Governor of the Province. He was mentioned in other reports for extracting the maximum amount of wood from a very difficult location. At this time he also showed that twisted fibre in Chir pine, a very damaging defect of this species, was a heritable character and not an effect of site conditions.

For the academic year 1924/25, while on leave pending retirement, he held the post of Silviculturist under Troup at the Imperial Forestry Institute, Oxford. In 1925 he married Chrystal Parsons, the grand-daughter of an Indian Army officer and decided to return to India — a momentous decision.

From 1925 until 1936 Champion was seconded to the Department of Education, Health and Lands of the Government of India and appointed Silviculturist at the Forest Research Institute, Dehra Dun. As Central Silviculturist he carried out research in a great variety of silvicultural projects, including natural forest regeneration and the establishment of plantations, as well as engaging in the preparation of yield tables and entomological problems.

He travelled extensively in India, and visited Burma and the Andaman Islands. In 1934/35 he was loaned to the Government of Ceylon to advise on forestry. From his experimental work and travelling he acquired an unparalleled knowledge of Indian forestry. During this period he also toured in the United States, Canada and the Far East. He visited most of the European forest research establishments and established himself as a forester of international repute.

In 1937 Champion returned to the United Provinces where he held the posts of Divisional Forest Officer, Conservator of Forests and Conservator of Working Plans acquiring experience of forest administration at senior level. He seemed destined to go to the top in the Indian Forestry Services, but in 1940 again changed the course of his career by accepting the Chair of Forestry at Oxford and the directorship of the Imperial Forestry Institute, as successor to Troup.

Champion's first notable achievement at Oxford was the establishment, in 1944, of an Honours School of Forestry. As Professor of Forestry he was *ex officio* Consultant Director to the Commonwealth Forestry Bureau and provided for the first Director, J.W.B. Sisam, and later Henry Ford Robertson, encouragement, advice and support to establish that most useful and efficient organisation. He also strengthened the scientific basis of teaching and research in forestry by the appointment of biological scientists. Between 1940 and 1959 he travelled extensively with forestry classes on the continent, on advisory missions and lecture tours to most of the British colonies, and to

attend international conferences. His experience in forestry was then world wide and he continued to make best use of it by writing and advising.

After his retirement in 1959 he was appointed Emeritus Professor in 1960 and until 1967 undertook further advisory missions and consulting assignments. In 1962/63 he was engaged by the Government of India to revise the silvicultural textbooks he had written when at Dehra Dun and had a similar assignment for the Government of Pakistan in 1963/64.

Champion published work, mainly in the Indian Forest Records, on every aspect of forestry, including research methods, statistics, working plans and ecology as well as silviculture. His interest in research methods is apparent from his *Silvicultural Research Manual for India, Vol. I: General*, and *Vol. II* (with I.D. Mahendru): *Statistical Code*, 1931. The results of his experimental work and his general observations were incorporated in a *Manual of Indian Silviculture* (with Sir Gerald Trevor) which has now been revised twice (in 1968 with S.K. Seth). In 1965, together with C.M. Khattak and S.K. Seth he prepared a similar manual for Pakistan. Of necessity, most of his time was devoted to matters of immediate and practical importance, but he was very concerned about the scientific basis of forestry and his *Forest Types of India and Burma* (1931, revised with S.K. Seth in 1968) is a major contribution to forest ecology.

Champion's work in India was acknowledged in 1941 by the award of Companion of the Order of the Indian Empire and his scientific achievements by an Oxford DSc in 1950. For his services to forestry in general he was made a Knight Bachelor in 1956. He was chairman of the Commonwealth Forestry Association from 1959 to 1961, president of the Society of Foresters of Great Britain in 1958 and 1959 and an honorary member of many national forestry societies. In 1969 he was given the Fernow Medal, awarded jointly by the American Forestry Association and the German Forestry Society.

Twenty classes of Oxford undergraduates and hundreds of foresters from every part of the world have been entertained in unique style by the Champions at Windrush. Some of us that were members of his earlier classes have further reason to be particularly grateful to them for many other kindnesses, among them dispensation to bring, what Chrystal Champion called our "probationary forest wives", on field tours.

Finally it is my privilege to put on record the immense contribution made by Chrystal Champion to the life and achievements of Harry Champion. She was keenly interested in his work, shared his fondness for travelling and perhaps persuaded him to return to India. It was a perfect match and good for forestry.

J.F. Hughes



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